GASTROINTESTINAL MECHANISMS FOR AVOIDING TOXICITY IN MAMMALIAN HERBIVORES

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Herbivory is the most common feeding strategy among mammals. The dietary niches of herbivores are largely determined by toxic plant secondary chemicals (PSCs) that alter the physiology of herbivores. Decades of ecological research have revealed that the detoxification systems of herbivores are adapted to metabolize PSCs and allow herbivores to consume toxic plants. However, the gastrointestinal tract may represent an earlier line of defense for herbivores. I have been studying interactions between the gastrointestinal tract of mammalian herbivores and PSCs. My research has focused on the desert woodrat (*Neotoma lepida*), which specializes on toxic creosote bush (*Larrea tridentata*).

I first investigated how digestive enzymes of woodrats respond to toxic PSCs. Creosote bush is covered in a phenolic-rich resin that inhibits digestive enzymes, which may in turn limit nutrient and energy availability to herbivores. I found that desert woodrats upregulate digestive enzyme activity when feeding on PSCs, presumably to overcome inhibition. These adaptations may be important for allowing woodrats to feed on toxic diets.

Next, I tested a longstanding hypothesis that gut microbes metabolize plant toxins and allow the ingestion of chemically defended plants. Woodrats have a semisegmented stomach that has been hypothesized to harbor a gut microbial community. I first characterized the microbial ecology of the woodrat gut by measuring the stomach pH of rodent species with and without stomach segmentation. Next, I conducted microbial

inventories of woodrat feces to demonstrate that woodrats harbor diverse and novel microbial communities, which they maintain in captivity. Further, I conducted inventories and various measurements on foregut contents to demonstrate that the foregut harbors a dense and active microbial community.

Finally, I tested interactions between PSCs and gut microbes. I found that PSCs greatly alter the microbial community structure of the woodrat gut. Additionally, I conducted a series of experiments consisting of whole-organism feeding trials, microbiome removal and microbial transplants to demonstrate that microbes can enhance tolerance to toxins.

This work demonstrates that the gastrointestinal tract is a site of adaptation and detoxification for mammalian herbivores. Further, it shows that microbial detoxification represents an accelerated mechanism by which herbivores may rapidly adapt to PSCs.

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CHAPTER 1

INTRODUCTION

Background

Herbivory is the most common feeding strategy among mammals (Price et al., 2012). Further, mammalian herbivores play critical roles in shaping ecosystem structure (Martin and Maron, 2012) and serve as essential resources to humans as livestock. Many plants produce plant secondary compounds (PSCs) that deter herbivory and pose significant challenges to mammalian herbivores (Dearing et al., 2005). In response, herbivores employ a variety of strategies to overcome these challenges (Freeland and Janzen, 1974). Decades of research have investigated mechanisms such as regulated intake or enhanced hepatic detoxification with the hopes of understanding how mammals are able to consume toxic plants (Dearing et al., 2005). Such an understanding may better inform agricultural practices, or be important for the conservation of herbivores facing novel and more potent PSCs brought about by environmental changes.

One largely overlooked site of adaptation to plant toxins has been the gastrointestinal tract, or gut. Plant toxins are known to influence the physiology of the gut by lowering digestibility, influencing gut transit time, or interacting with the microbiota (reviewed in Karasov and Douglas 2013). Research on herbivorous insects has revealed that the gut is an important organ for allowing them to feed on toxic plants (Karasov and

Douglas, 2013). However, few studies have investigated similar questions in mammalian herbivores. One exception is work investigating efflux transporters located in the guts of specialist mammalian herbivores, which act to limit the absorption of toxic compounds (Dearing et al., 2005).

My dissertation has investigated the gastrointestinal mechanisms allowing mammalian herbivores to feed on toxic plants. I have focused on woodrats (*Neotoma spp.*), small herbivorous rodents that tend to specialize on toxic plants. My results show that the gut is a site of adaptation that allows mammalian herbivores to consume chemically defended plants. Specifically, mammalian herbivores have adapted through increasing digestive enzyme activities and harboring communities of microbes that aid in metabolizing toxins. Interestingly, my work suggests that microbial communities can rapidly confer tolerance to toxins through microbial transplants, which may represent a frontier for the future of agriculture and conservation biology.

Intestinal Enzymes

Many plants contain defensive compounds that act to bind digestive enzymes, thus inhibiting digestion and limiting nutrient availability to herbivores (Min et al., 2003). To overcome this challenge, numerous insect herbivores increase enzyme activity or produce variants of enzymes that are tolerant to inhibition (Jongsma and Bolter, 1997). However, such adaptations have not been documented in mammalian herbivores.

In Chapter 2, I investigated whether the digestive enzymes of Bryant's woodrat (*Neotoma bryanti*) respond to plant toxins. Woodrats were fed a diet containing 2% resin extracted from creosote bush (*Larrea tridentata*). Creosote resin is a phenolic-rich mixture that is known to inhibit digestive enzymes (Rhoades, 1977). I found that

woodrats that were fed creosote exhibited higher amylase and chymotrypsin activities. Moreover, I found that woodrats produced a variant of a protein-digesting enzyme that was more resistant to inhibition by creosote when the animals were fed the diet containing PSCs. This research demonstrates that similar to insect herbivores, mammalian herbivores exhibit digestive adaptations that may allow them to consume toxic diets.

Microbial Ecology of the Woodrat Gut

It has long been hypothesized that gut microbes may also aid herbivores in consuming chemically defended plants (Freeland and Janzen, 1974). Recent research has shown that gut microbes influence many aspects of animal physiology, such as immune function, nutrition, and even behavior (McFall-Ngai et al., 2013). However, empirical studies investigating whether the gut microbiota allow mammals to feed on toxic plants have been lacking.

Before addressing this gap in knowledge, I first characterized the microbial ecology of the woodrat gut. A recent, large interspecies study of mammals demonstrated that gut microbial community composition is determined by the gut anatomy (foregut- versus hindgut-fermenting), diet, and evolutionary history of the mammalian host (Ley et al., 2008). However, this study included very few rodents. Woodrats are especially interesting given that they have a semisegmented stomach with a foregut chamber that has been hypothesized to house a microbial community for over a century (Toepfer, 1891; Carleton, 1973), yet this idea lacks empirical support. In Chapter 3, I investigated how stomach segmentation might create a suitable environment for hosting gut microbes by comparing the gut pH of various rodent species with and without stomach

segmentation. I found that rodents with segmented stomachs had a larger pH gradient between the foregut and gastric stomach chambers, and achieved an overall lower gastric pH. Chapter 4 presents the results of microbial inventories from the feces of Bryant's woodrat. I found that the community composition of woodrat feces was more similar to foregut-fermenting mammals rather than other, closely related hindgut-fermenting rodents. Together, these results lent support to the century-old hypothesis that woodrats house a dense microbial community.

In Chapter 5, I tracked microbial diversity of woodrats before and after 6 months in captivity to monitor changes in diversity. Woodrats maintained a majority of the microbial species that they hosted in the wild. These results offered evidence that this system was tractable even when animals were brought in to captivity.

Finally, I conducted a thorough investigation in to the microbial ecology of the woodrat gut. I measured the size, pH, bacterial cell density, concentrations of microbial metabolites, and digesta transit time of various chambers of the woodrat gut. Further, I conducted microbial inventories of these chambers. Chapter 6 shows that woodrats indeed house a foregut microbial community, with bacterial cell density and metabolite concentrations on par with the cecum, a well-known microbial chamber. Further, microbial communities varied markedly between gut chambers. Overall, I have shown that woodrats house a foregut microbial community that they retain in captivity. These results laid the groundwork for investigating interaction between plant toxins and gut microbes in this wild mammalian herbivore.

Interactions Between PSCs and Gut Microbes

With a more thorough understanding of the microbial ecology of the woodrat gut, I was able to investigate how plant toxins impact gut microbial communities. For decades, it has been hypothesized that PSCs sculpt microbial communities (Freeland and Janzen, 1974). I conducted a feeding trial to demonstrate that creosote toxins largely reshape gut microbial communities and that these responses depend on previous ecological and evolutionary experience with PSCs. These data (Chapter 7) suggested that the microbiota had adapted to creosote toxins and led me to hypothesize that microbes allow woodrats to consume PSCs. Therefore, I conducted a series of experiments consisting of whole-animal feeding trials, microbiome removal through antibiotics, and microbial transplants to show that gut microbes indeed facilitate the ingestion of toxic plants. Further, I used metagenomic and metabolomic approaches to measure the function of the gut microbiota. I found that a microbial gene, aryl alcohol dehydrogenase, is significantly more abundant when woodrats are fed creosote toxins. This work, presented in Chapter 8, has revealed that microbes can enhance host tolerance to toxins and thus, expand the dietary niche breadth of wild mammalian herbivores.

Future Directions

My research has shown that adaptations within the gastrointestinal tract allow herbivorous mammals to consume toxic plants. Future work could investigate the mechanisms that allow digestive enzymes to become resistant to inhibition by phenolics (sequence changes, posttranslational modifications, etc.). Additionally, other studies could investigate the generality of this finding in other herbivores or various classes of toxins. This work has also demonstrated that gut microbes facilitate the ingestion of

chemically defended plants and that this ability can be transferred. Future studies could transplant the microbiota of wild herbivores into agricultural herbivores facing toxic challenges. These findings could greatly improve agricultural practices.

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CHAPTER 2

INDUCED AND CONSTITUTIVE RESPONSES OF DIGESTIVE ENZYMES TO PLANT TOXINS IN AN HERBIVOROUS MAMMAL

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RESEARCH ARTICLE

Induced and constitutive responses of digestive enzymes to plant toxins in an herbivorous mammal

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SUMMARY

Many plants produce plant secondary compounds (PSCs) that bind and inhibit the digestive enzymes of herbivores, thus limiting digestibility for the herbivore. Herbivorous insects employ several physiological responses to overcome the anti-nutritive effects of PSCs. However, studies in vertebrates have not shown such responses, perhaps stemming from the fact that previously studied vertebrates were not herbivorous. The responses of the digestive system to dietary PSCs in populations of Bryant's woodrat (*Neotoma bryanti*) that vary in their ecological and evolutionary experience with the PSCs in creosote bush (*Larrea tridentata*) were compared. Individuals from naïve and experienced populations were fed diets with and without added creosote resin. Animals fed diets with creosote resin had higher activities of pancreatic amylase, as well as luminal amylase and chymotrypsin, regardless of prior experience with creosote. The experienced population showed constitutively higher activities of intestinal maltase and sucrase. Additionally, the naïve population produced an aminopeptidase-N enzyme that was less inhibited by creosote resin when feeding on the creosote resin diet, whereas the experienced population constitutively expressed this form of aminopeptidase-N. Thus, the digestive system of an herbivorous vertebrate responds significantly to dietary PSCs, which may be important for allowing herbivorous vertebrates to feed on PSC-rich diets.

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Key words: digestive enzymes, plant–herbivore interactions, plant secondary chemicals.

INTRODUCTION

Plants produce a wide array of plant secondary compounds (PSCs) to deter feeding by herbivores (Dearing et al., 2005). Many of these compounds, such as tannins or flavonoids, may bind to plant protein, preventing its digestion, and thus lowering the nutrient availability to the animal (Min et al., 2003). Additionally, if these compounds are present in high enough concentrations, they may bind and inhibit digestive enzymes produced by the herbivore, further limiting nutrient availability (Min et al., 2003).

To counteract the effects of PSCs on digestive enzymes, herbivores may employ one or more strategies. First, herbivores may alter the environment of their gut to prevent protein precipitation, perhaps through an alkaline-biased pH (Berenbaum, 1980). Second, herbivores can maintain baseline activity through increased synthesis of digestive enzymes to compensate for activity lost to inhibition (Jongsma and Bolter, 1997). Additionally, herbivores might produce variants of digestive enzymes that are less inhibited by PSCs (Jongsma and Bolter, 1997). Such responses have been well documented in herbivorous insects (Jongsma and Bolter, 1997). In contrast, many studies on terrestrial vertebrates have documented little response of digestive enzymes to PSCs, with some even exhibiting decreases in the activities of intestinal or pancreatic enzymes (Ahmed et al., 1991; Glick and Joslyn, 1970; Longstaff and McNab, 1991; Mariscal-Landin et al., 2004; van Leeuwen et al., 1995).

The differences in the responses observed in insects *versus* vertebrates may stem from the fact that the vertebrates tested (domestic rats, pigs and chickens) are all evolutionarily naïve to high concentrations of PSCs as none are typically herbivorous, whereas all the insects examined are herbivorous. The physiological responses of the digestive systems of herbivorous insects are thought to have evolved with the defense compounds of their host plants (Jongsma and Bolter, 1997). Likewise, the detoxification systems of vertebrate herbivores are believed to have evolved in response to the ingestion of PSCs (Dearing et al., 2005; Freeland and Janzen, 1974). Thus, investigations in wild herbivores may give better insight into the physiological and evolutionary responses of the digestive systems of vertebrates to PSCs.

We investigated the digestive responses of vertebrates to PSCs in a wild mammalian herbivore. Bryant's woodrat (*Neotoma bryanti* Merriam 1887) is a small, herbivorous mammal found primarily in California, USA (Patton et al., 2007). *Neotoma bryanti* feeds primarily on cactus (*Opuntia occidentalis*) and sage (*Salvia* spp.) in the coastal regions of its range (Atsatt and Ingram, 1983), whereas it consumes creosote bush (*Larrea tridentata*) in desert habitats. Creosote bush is interesting from a dietary perspective because it is a relatively recent dietary addition given that it has only been present in the southwestern deserts of the USA since the end of the last glacial period (*ca.* 17,000 years ago) (Hunter et al., 2001). Thus, this *Neotoma* species has coastal populations that are evolutionarily and ecologically naïve to creosote bush ('naïve population'), as well as desert populations that have had >10,000 years of contact with creosote bush and its PSCs ('experienced population'). These populations vary in the types of PSCs they encounter in the wild. The naïve population feeds mainly on plants that produce low-molecular-weight defense compounds that are unlikely to bind

dietary or enzymatic proteins (Abreu et al., 2008; Atsatt and Ingram, 1983; Stintzing and Carle, 2005). Alternatively, creosote bush leaves produce a complex resin consisting of various phenolics, flavonoids and saponins (Mabry et al., 1977), chemical classes that have been shown to bind and inhibit digestive enzymes (Cheeke, 1971; Fontana Pereira et al., 2011). Although interactions between digestive enzymes and specific compounds in creosote resin remain unstudied, creosote resin does bind protein and inhibit proteolytic enzymes (Rhoades, 1977). Because of the differences in plant chemistry that each of these populations naturally consume, their physiological responses to protein-binding PSCs are expected to vary.

We collected individuals from each of these populations and fed them diets containing creosote bush resin. We investigated for the presence of induced responses, predicting that when feeding on creosote resin individuals would: (1) increase their gut pH, (2) increase the mass-specific activity of digestive enzymes and (3) produce variants of digestive enzymes that are less inhibited by creosote resin. Additionally, we examined for constitutive responses, in which we predicted the experienced population to maintain: (4) a higher gut pH, (5) higher mass-specific enzyme activities and (6) a constant production of enzyme variants that were less inhibited by creosote resin in comparison with the naïve population.

MATERIALS AND METHODS

Animal collection and maintenance

Individuals of the naïve population were collected in July 2006 from Casper's Wilderness Area, CA (33°31′N, 117°33′W). Individuals of the experienced population were collected in April 2009 outside Palm Desert, CA (33°68′N, 116°36′W), in the Sonoran Desert. All animals were trapped with Sherman live traps. Woodrats were transported to the University of Utah Department of Biology Animal Facility and housed in individual cages (48×27×20 cm) under a 12 h:12 h light:dark cycle, with 28°C ambient temperature and 20% humidity.

Dietary treatments and diet preparation

Prior to experimentation, animals were maintained on a diet of pelleted high-fiber rabbit chow (Teklad formula 2031, Harlan Laboratories, Madison, WI, USA). During experimentation, animals were fed the same rabbit chow formulation except in a powdered form to prevent caching of food. Five individuals from each population served as control animals and were fed powdered rabbit chow in cages with feeder-hoods for 8 days. Nine woodrats (four from the naïve population, five from the experienced population) were fed the control diet for 3 days followed by the same diet with increasing amounts of creosote resin (1 and 2% creosote resin for 2 and 3 days, respectively). This protocol was used to permit time for the induction of digestive enzymes (Deren et al., 1967). A diet with 2% creosote is tolerated without body mass loss in naïve animals. Body mass and dry matter intake (DMI) of all animals was measured daily. DMI was calculated as the difference between the amount of food presented and what remained each day, after samples were dried at 50°C for 3 days.

To prepare creosote resin diets, creosote leaves were collected from trapping sites and frozen at –20°C prior to resin extraction. Resin was extracted by soaking leaves in acetone (1:6, wet leaf mass:volume solvent) for 45 min. Solvent and resin were filtered (Whatman filter paper grade 1) to remove large particles and evaporated using a rotovap until the resin was highly viscous, at which point it was transferred to a vacuum pump for 48 h to remove any remaining acetone. Extracted resin was stored at –20°C prior to use.

Creosote diet was prepared by dissolving the appropriate amount of resin in a volume of acetone equal to 25% of the dry mass of ground rabbit chow to which it was added. Control diet (0%) was prepared by adding an identical ratio of acetone, without creosote resin. Acetone was evaporated from all diets in a fume hood, and complete evaporation was confirmed gravimetrically.

Following diet treatments, animals were euthanized under CO_2 and immediately dissected. Pancreatic tissue was removed, weighed and frozen on dry ice. The luminal contents of the small intestine were removed, pH was measured with an Omega Soil pH electrode (PHH-200) and then contents were frozen. The intestine was then flushed with ice-cold saline, cut in half length-wise, weighed and frozen. Tissues were kept at –80°C until analysis.

Pancreatic and luminal enzyme assays

Activities of pancreatic and luminal amylase were measured by modification of the 3,5-dinitrosalicylate method (Dahlqvist, 1962). For pancreatic amylase, several pieces of pancreas were thawed and homogenized for 30 s using 10 ml g^{-1} tissue of amylase homogenizing buffer [5 mmol l^{-1} phosphate buffer, pH 6.9, containing 7 mmol l^{-1} NaCl, 3 mmol l^{-1} taurocholic acid, 0.27% (w/v) Triton X-100, 1 mmol l^{-1} benzamidine and 2 mmol l^{-1} hydrocinnamic acid]. For luminal amylase, lumen contents of the intestine were vortexed in 10 parts of amylase homogenizing buffer and then centrifuged for 2 min at 7000 ***g*** to collect the supernatant. Diluted 100 µl aliquots of either pancreas homogenate or luminal supernatant were incubated with 100 µl of 2% potato starch (Sigma-Aldrich S2630, St Louis, MO, USA) at 37°C for 3 min. The reaction was terminated by the addition of 200 µl dinitrosalicylate reagent. The tubes were immersed in boiling water for 10 min and cooled with tap water. Blank samples contained exactly the same reagents, but dinitrosalicylate was added before substrate to deactivate enzymes and prevent reaction, and then were handled in the same way as other samples. Aliquots of 150 µl were transferred to 96-well plates, and absorbance at 530 nm was determined using a BioTek PowerWave HT microplate spectrophotomer (Broadview, IL, USA).

For analysis of pancreatic chymotrypsin and trypsin, several pieces of pancreatic tissue were homogenized for 30 s using 10 ml g^{-1} tissue of chymotrypsin/trypsin homogenizing buffer [50 mmol l^{-1} Tris/HCl buffer, pH 8.2, containing 3 mmol l^{-1} taurocholic acid and 0.27% (w/v) Triton X-100]. To activate zymogens, homogenate samples were incubated with 0.3% enterokinase (Sigma-Aldrich E0632) in 50 mmol Tris/HCl buffer (pH 8.2) containing 20 mmol l^{-1} $CaCl_2$ for 20 min at 37°C. Preliminary trials indicated that this treatment gave reproducible maximal activation of the proteolytic zymogens. Samples were centrifuged for 2 min at 7000 ***g*** to remove a white suspension that sometimes appears in solution. Chymotrypsin activities were measured by the amount of p-nitroaniline released by hydrolysis when incubating 160 µl of homogenate supernatant and 800 µl of 1 mmol l^{-1} N-glutaryl-l-phenylalanine-p-nitroanilide (GPNA) solution at pH 7.6 for 10 min at 37°C. Aliquots of 16 µl supernatant mixed with 144 µl distilled water were assayed to measure trypsin activity using 800 µl 1 mmol l^{-1} benzoyl-arginine-p-nitroanilide (DL-BAPNA) solution as substrate at pH 8.2 for 10 min at 37°C. For both chymotrypsin and trypsin assays, reactions were terminated with 160 µl of 30% acetic acid solution. Blank samples contained exactly the same reagents but acetic acid was added before the substrate to deactivate enzymes and prevent reaction, and then they were handled in the same way as other samples. The liberated amounts of p-nitroaniline were estimated by transferring 200 µl of the final reaction to a 96-well

plate and reading the absorbance at 410 nm (Erlanger et al., 1961), and enzyme activity was calculated using a p-nitroaniline standard curve.

Luminal chymotrypsin and trypsin activities were measured similarly. Lumen contents were vortexed in 10 parts of chymotrypsin/trypsin homogenizing buffer and then centrifuged for 2 min at 7000 ***g*** to collect the supernatant. Further activation by enterokinase was not required for luminal contents. For chymotrypsin, 160 µl of supernatant was added to 800 µl of 1 mmol l^{-1} GPNA. For trypsin, 80 µl of supernatant and 80 µl of distilled water were added to 800 µl of 1 mmol l^{-1} DL-BAPNA. All other steps were identical to the technique for measuring pancreatic enzyme activities.

Intestinal enzyme assays

Intestinal enzyme assays were carried out on the proximal half of the small intestine. We assayed disaccharidase (maltase, sucrase) activity using a modification of a previously developed colorimetric method (Dahlqvist, 1984). Briefly, tissues were thawed and homogenized in 350 mmol l^{-1} mannitol in 1 mmol l^{-1} N-2-hydroxyethylpiperazine-N′-2-ethanosulfonic acid (Hepes)-KOH, pH 7.0. Intestinal homogenates (30 µl) diluted with 350 mmol l^{-1} mannitol in 1 mmol l^{-1} Hepes-KOH were incubated with 30 µl of 56 mmol l^{-1} maltose or 56 mmol l^{-1} sucrose in 0.1 mol l^{-1} maleate and NaOH buffer, pH 6.5, at 37°C for 20 min. Next, 400 µl of a stop-develop reagent (GAGO-20 glucose assay kit, Sigma-Aldrich) was added to each tube, vortexed and incubated at 37°C for 30 min. Lastly, 400 µl of 6 mol l^{-1} H_2SO_4 was added to each tube to stop the reaction. Several 200 µl aliquots of the final reaction were transferred to a 96-well plate, and the absorbance was read at 540 nm.

We used L-alanine-p-nitroanilide as a substrate for aminopeptidase-N. To start the reaction we added 10 µl of the homogenate to 1 ml of assay mix (2.0 mmol l^{-1} L-alanine-p-nitroanilide in one part of 0.2 mol l^{-1} NaH_2PO_4/Na_2HPO_4 buffer no. 1, pH 7 and one part of deionized H_2O). The reaction solution was incubated for 20 min at 37°C and then the reaction was terminated with 3 ml of ice-cold 2 mol l^{-1} acetic acid. Several 200 µl aliquots of the final reaction were transferred to a 96-well plate, and absorbance was measured at 384 nm.

Inhibition of enzyme activity by creosote resin

We added creosote resin to enzyme activity reactions to monitor enzyme inhibition. We were unable to measure the inhibition of maltase and sucrase activity, as the stop-develop reagent used for these enzyme assays utilizes the purified enzymes glucose oxidase and peroxidase. We could not design a protocol that inhibited maltase or sucrase activity, but did not also inhibit the color-developing enzymes. For all other enzymes (amylase, trypsin, chymotrypsin and aminopeptidase-N), tissues were incubated with a series of concentrations of creosote resin directly following homogenization. Resin concentrations were determined specifically for each enzyme based on the concentrations that gave significant inhibition of enzyme activity. Resin was dissolved in 85% methanol, added to tissue homogenates and allowed to sit for 10 min. Control tubes contained only tissue homogenate and 85% methanol. Tissue homogenates were centrifuged for 1 min at 7000 ***g*** to remove a suspension created by the resin solution. Supernatants were used to run enzyme assays as described above. Relative enzyme activity was calculated by dividing the activity of resin-treated homogenates by the activity of control homogenates.

Statistics

Body mass, small intestinal pH and DMI were compared using separate two-way ANOVAs, with population and diet treatment as main effects. Additionally, absolute organ masses were compared using diet and population as main effects and body mass as a covariate. Enzyme activities were compared with two-way ANOVAs, using population and diet treatment as main effects, as well as investigating DMI as a covariate. If the covariate of DMI was insignificant, it was removed from the model. Significant differences between treatment groups were detected using Tukey's honestly significant difference (HSD) test. Relative enzyme activity was compared using repeated-measures ANOVA with population and diet treatment as main effects. Statistical analyses were conducted in JMP (SAS Institute, 2010).

RESULTS

Body and organ masses, intestinal pH and food intake

Individuals from the naïve population were larger than those from the experienced population (Table 1). There were no population, diet treatment or interaction effects on the pH of small intestinal contents (Table 1). Because of a limited amount of digesta, intestinal pH for one animal was not measured. Creosote caused animals to consume significantly less food, and this decrease was more exaggerated in the naïve population (Table 1). However, DMI was not a significant covariate in analysis of any enzyme activity, and so it was removed from all analyses. There were no significant effects of diet or population on organ masses when controlling for body mass (Table 1).

Pancreatic and luminal enzyme activities

The response of pancreatic and luminal enzymes to creosote resin varied across enzymes. Addition of creosote resin to the diet significantly increased mass-specific amylase activity of both populations (Table 2, Fig. 1A). Naïve individuals feeding on creosote showed 4.3× higher amylase activity in the pancreas compared with controls, and experienced individuals showed 2.6× higher activity. However, this response did not differ between populations, as no significant interaction between population and diet treatment was observed (Table 2). There were no significant effects of population or diet treatment on pancreatic chymotrypsin or trypsin activity (Table 2, Fig. 1B,C). Two of the luminal enzymes assayed (amylase and chymotrypsin) showed higher activity in individuals fed creosote (Table 2, Fig. 2A,B). Because of a limited amount of digesta, we were unable to measure luminal trypsin activity for one animal. There was no significant effect of population or diet on luminal trypsin activity (Table 2, Fig. 2C).

Intestinal enzyme activities

The experienced population exhibited 1.8× higher maltase activity and 1.5× higher sucrase activity compared with the naïve population (Table 2, Fig. 3A,B). However, diet did not significantly alter disaccharidase activity in either population (Table 2). Additionally, there were no significant effects of population or diet treatment on intestinal aminopeptidase-N activity (Table 2, Fig. 3C).

Inhibition of enzyme activity by creosote resin

For all enzymes, creosote resin significantly inhibited enzyme activity (resin concentration effect: $P<0.0001$ for all enzymes; Fig. 4, supplementary material Fig. S1). For amylase, chymotrypsin and trypsin, there were no effects of population or diet treatment on enzyme inhibition by creosote resin *in vitro* (supplementary material Fig. S1). Relative aminopeptidase-N activity was not significantly

Table 1. (A) Means ±1 s.e.m. and (B) summary of ANOVAs for body mass, intestinal pH and dry matter intake (DMI), and ANCOVAs for organ masses

	Naïve		Experienced	
A) Variable	Control	Creosote	Control	Creosote
Sample size	5	4	5	5
Body mass (g)	161.5±11.9	159.1±14.4	127.0±12.0	120.3±9.1
Intestinal pH	7.98±0.08	7.99±0.16	7.80±0.09	7.94±0.10
DMI ($g\,d^{-1}$)	10.59±0.48	6.99±0.41	9.49±0.41	8.92±0.63
Pancreas mass (g)	0.37±0.03	0.33±0.06	0.30±0.03	0.23±0.03
Intestine mass (g)	3.26±0.18	2.99±0.24	2.29±0.15	2.36±0.21

B) Source of variation	*F*	d.f.	*P*
Body mass			
Population	9.61	1,15	**0.007**
Diet	0.15	1,15	0.71
Interaction	0.03	1,15	0.86
Intestinal pH			
Population	1.06	1,14	0.32
Diet	0.44	1,14	0.52
Interaction	0.45	1,14	0.51
DMI			
Population	0.66	1,15	0.43
Diet	16.99	1,15	**<0.001**
Interaction	8.95	1,15	**0.009**
Pancreas mass			
Population	0.46	1,14	0.51
Diet	1.38	1,14	0.26
Interaction	0.15	1,14	0.70
Covariate (body mass)	3.73	1,14	0.07
Intestine mass			
Population	3.99	1,14	0.07
Diet	0.10	1,14	0.75
Interaction	1.57	1,14	0.23
Covariate (body mass)	10.26	1,14	**0.006**

Significant differences are in bold.

altered by diet treatment in the experienced population ($F_{1,8}$=0.0008, P=0.98; Fig. 4A). In the naïve population, though, individuals feeding on creosote produced an aminopeptidase-N enzyme that was less inhibited ($F_{1,7}$=14.56, P=0.006; Fig. 4B), and very similar to that of the experienced population.

DISCUSSION

Herbivores can mediate the inhibitory impact of PSCs on their digestive process through several mechanisms. They can alter gut pH, increase enzyme activities or produce variants of digestive enzymes less subject to inhibition. These responses have not been extensively documented in vertebrate herbivores. In this study, we compared evolutionarily and ecologically naïve herbivores with experienced ones to investigate whether experience played a role in the ability to respond to PSCs. Indeed, the consumption of PSCs alters digestive enzyme activity and inhibition rates in a wild mammalian herbivore. How these responses varied as a function of the particular enzyme as well as the animal's experience with PSCs and the organismal implications are described.

A highly alkaline gut pH is thought to be an important mechanism by which larval insects avoid inhibition of digestive enzymes (Berenbaum, 1980). However, no significant differences in the pH of intestinal contents between populations or diet treatments were observed. Vertebrates may be limited in their ability to increase gut pH as a defense against PSCs, as their digestive enzymes usually operate optimally within pH ranges of 6 to 7.5 (Blair and Tuba, 1963; Maze and Gray, 1980), whereas some insect digestive enzymes can perform optimally up to a pH of 12 (Wolfson and Murdock, 1990). However, it is noteworthy that the intestinal pH of *N. bryanti* (~7.9) is far higher than that found in laboratory rodents (pH 5–6) (McConnell et al., 2008). Further surveys of intestinal pH in rodents with different dietary habits may be warranted.

Pancreatic amylase activity increased in *N. bryanti* individuals feeding on creosote resin. Previous experiments on digestive enzymes in terrestrial vertebrates have not shown changes in mass-specific activity of pancreatic amylase, but showed hypertrophy of the pancreas after feeding on tannins for 4 weeks, resulting in greater activity summed across the pancreas (Ahmed et al., 1991). In our study, diet had no effect on pancreas size, perhaps because creosote diet treatments were ingested for only 5 days. The increase in mass-specific amylase activity seen in woodrats feeding on creosote resin may represent an acute response to toxins, and longer exposure might result in pancreatic hypertrophy along with the loss of any mass-specific differences in amylase activity.

Luminal activities of pancreatic enzymes may be a better measure than pancreatic activities by which to investigate physiological responses to enzyme-binding toxins. Prior to acting on nutrients, enzymes stored within pancreatic tissue must be secreted into the intestinal lumen, as well as activated by enterokinase (in the case of trypsin and chymotrypsin) (Stevens and Hume, 2004). Woodrats feeding on creosote resin showed higher amylase and chymotrypsin activity in the intestinal lumen compared with control-fed animals. These data, coupled with those for pancreatic enzyme activity, allow inference into the storage and release of pancreatic enzymes in response to dietary toxins. Based on the lumen data, when fed creosote resin, woodrats appear to increase the secretion of

Table 2. ANOVA results for enzyme activities

Enzyme	*F*	d.f.	*P*
Pancreatic enzyme activities			
Amylase			
Population	1.04	1,15	0.32
Diet	16.13	1,15	**0.001**
Interaction	1.39	1,15	0.26
Chymotrypsin			
Population	0.09	1,15	0.77
Diet	0.06	1,15	0.81
Interaction	0.11	1,15	0.74
Trypsin			
Population	0.0004	1,15	0.98
Diet	0.02	1,15	0.88
Interaction	0.43	1,15	0.52
Luminal enzyme activities			
Amylase			
Population	0.05	1,15	0.82
Diet	6.18	1,15	**0.025**
Interaction	0.02	1,15	0.88
Chymotrypsin			
Population	0.001	1,15	0.97
Diet	9.07	1,15	**0.009**
Interaction	1.35	1,15	0.26
Trypsin			
Population	2.26	1,14	0.15
Diet	2.87	1,14	0.11
Interaction	0.23	1,14	0.64
Intestinal enzyme activities			
Maltase			
Population	5.55	1,15	**0.032**
Diet	0.001	1,15	0.97
Interaction	0.34	1,15	0.57
Sucrase			
Population	4.78	1,15	**0.045**
Diet	0.51	1,15	0.48
Interaction	0.03	1,15	0.86
Aminopeptidase-N			
Population	0.11	1,15	0.74
Diet	0.10	1,15	0.76
Interaction	0.12	1,15	0.90

Significant differences are in bold.

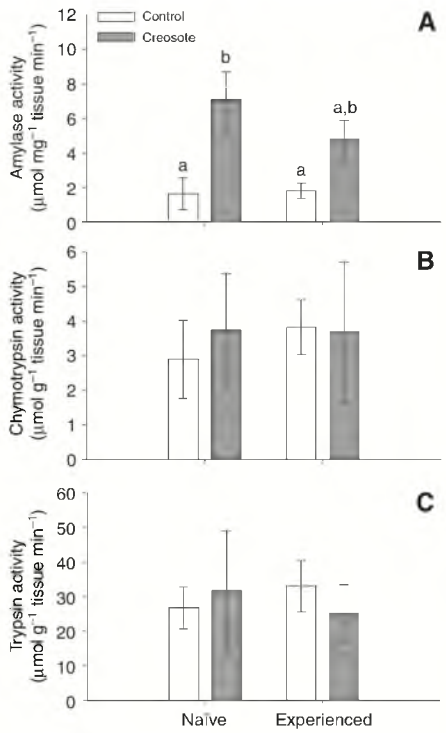

Fig. 1. Mean mass-specific activities of (A) amylase, (B) chymotrypsin and (C) trypsin within pancreatic tissue of woodrats with different ecological and evolutionary experience with, as well as short-term exposure to, PSCs. Bars represent ±1 s.e.m. Different letters above bars indicate significant differences between treatments (Tukey's HSD).

chymotrypsin into the intestine. However, the lack of difference in pancreatic chymotrypsin activity suggests that the pancreas maintains an even and balanced level of stored chymotrypsinogen (the precursor to chymotrypsin). Thus, dietary toxins may increase the turnover of this enzyme within the pancreas. In contrast, the increases in both pancreatic and luminal amylase activity suggest that in response to toxins, the pancreas not only secretes more amylase, but also stores excess amylase, which may be lost if the pancreas undergoes hypertrophy following long-term exposure to dietary toxins as in other vertebrates (Ahmed et al., 1991).

Additionally, these results suggest that modulation of the secretion rates of pancreatic enzymes may be an important mechanism for physiological responses. Secretion of pancreatic enzymes is tightly regulated by the gastrointestinal hormones cholecystokinin and secretin (Hadley and Levine, 2007). In fact, trypsin inhibitors found in soybeans induce secretion of cholecystokinin from intestinal tissue, which then induces trypsin production and secretion (Savelkoul et al., 1992). However, many studies investigating responses of digestive enzymes to changes in diet composition (usually nutrients) measure activity only within pancreatic tissue and not the lumen (Ciminari et al., 2001; Kohl et al., 2011). Future studies should integrate enzyme activities of pancreatic tissue with that of the intestinal lumen when investigating responses of pancreatic enzymes to changes in dietary substrates or toxins.

Activities of intestinal carbohydrases showed constitutive differences between populations such that the naïve population had lower activity than the experienced population. This is surprising, given that activities of maltase and sucrase in wild and laboratory rodents are quickly and drastically modulated in response to changes in diet (Karasov and Hume, 1997). Modulation of intestinal disaccharidases seems to be dependent on glucocorticoid signals released by the adrenal gland (Lebenthal et al., 1972). It could be that signaling pathways do not exist to convey information on dietary toxin presence to promoters of disaccharidase expression. If true, experienced animals may have solved the problem of maltase and sucrase inhibition through higher constitutive activity of these enzymes to deal with a natural diet rich in enzyme inhibitors.

Woodrats seemed to respond to inhibition of aminopeptidase-N by altering the enzyme variant produced, rather than modulating activity of this enzyme. In naïve populations feeding on a control diet, the aminopeptidase-N enzyme was significantly inhibited by the addition of creosote. However, when naïve individuals were fed a creosote-containing diet, they produced an aminopeptidase-N enzyme that was less inhibited by creosote and showed inhibition rates similar to those of all experienced individuals, regardless of diet. These results are similar to those in insects showing that after

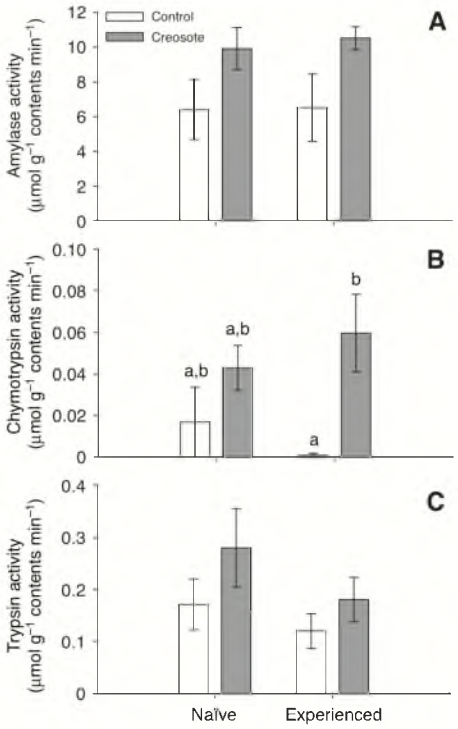

Fig. 2. Mean mass-specific activities of (A) amylase, (B) chymotrypsin and (C) trypsin from intestinal lumen contents of woodrats with different ecological and evolutionary experience with, as well as short-term exposure to, PSCs. Bars represent ±1 s.e.m. Different letters above bars indicate significant differences between treatments (Tukey's HSD).

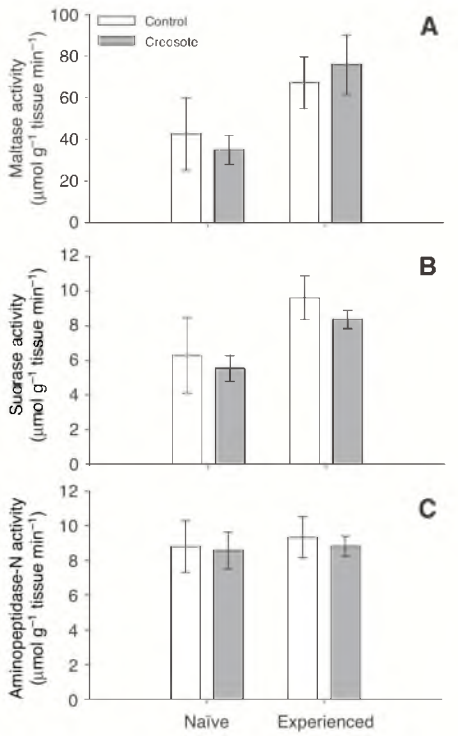

Fig. 3. Mean mass-specific activities of (A) maltase, (B) sucrase and (C) aminopeptidase-N from intestinal tissue of woodrats with different ecological and evolutionary experience with, as well as short-term exposure to, PSCs. Bars represent ±1 s.e.m.

feeding on plants containing protease inhibitors, insects produce variants of proteases that are insensitive to inhibition (Jongsma et al., 1995). This change in variants is achieved through transcriptional induction of insensitive protease genes belonging to complex, multigene families (Bown et al., 1997). However, the aminopeptidase-N gene is only represented by one copy in the mouse genome (Waterson et al., 2002). Still, the possibility of gene duplication and differentiation within *N. bryanti*, and differential transcriptional regulation between populations, cannot be discarded, as new gene families can arise between closely related mammals (Demuth et al., 2006). Another possible mechanism of inducing enzymes tolerant to protease inhibitors is through post-translational modifications. For example, protein glycosylation significantly decreases binding by tannins (Sarni-Manchado et al., 2008). Future work addressing these mechanisms will enlighten whether *N. bryanti* has convergently or uniquely solved the challenge of protease inhibition.

Functional implications

It is interesting that naïve populations are able to physiologically respond to a completely novel toxin, creosote resin. Naïve populations of *N. bryanti* prefer to feed on plants that largely contain low-molecular-weight PSCs (Abreu et al., 2008; Atsatt and Ingram, 1983; Stintzing and Carle, 2005) that are unlikely to inhibit digestive enzymes. However, coastal live oak (*Quercus agrifolia*), which is rich in phenolics and tannins, also grows within the native habitat of naïve *N. bryanti* (Atsatt and Ingram, 1983). Naïve *N. bryanti* are able to ingest and maintain body mass on a diet consisting only of oak (Skopec et al., 2008), but do not choose to consume oak when other food sources are available (Atsatt and Ingram, 1983). The physiological mechanisms by which naïve *N. bryanti* are able to respond to novel creosote toxins may be due to an ability to detect and respond to the phenolics and tannins present in both oak and creosote. These generic responses might allow naïve *N. bryanti* to feed on oak in times of intense intraspecific competition or during low abundance of preferred plant species.

Overall, the responses exhibited by digestive enzymes to creosote resin are expected to facilitate digestion and nutrient assimilation of poor-quality diets. We do not have digestibility data from *N. bryanti* feeding on control or creosote diets. However, the possibility exists that, because of the long-term exposure of desert populations of *N. bryanti* to creosote, their combination of both induced and constitutive responses might allow increased digestibility of creosote rich diets over naïve populations. Indeed, creosote resin does not alter energy digestibility in another experienced species, the desert woodrat (*N. lepida*) (Mangione et al., 2004). Digestibility trials comparing populations of *N. bryanti* on day 1 of a creosote diet treatment (presumably before induction of digestive enzymes) may also be necessary to address whether the constitutive differences found in this study are functionally significant.

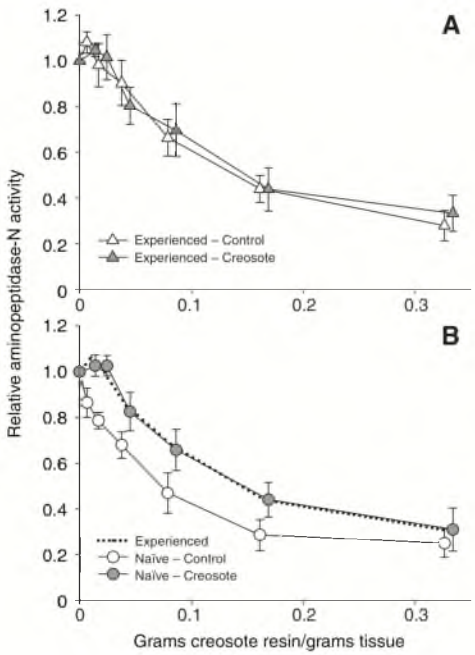

Fig. 4. Relative aminopeptidase-N activity with increasing creosote resin concentrations added *in vitro* for the (A) experienced population and (B) naïve population with differing dietary treatments. Dotted line in B represents the mean of all experienced individuals for comparison. Bars represent ±1 s.e.m.

The results presented in this study are the first to show increases in digestive enzyme activities of terrestrial vertebrates in response to plant toxins, independent of changes in organ mass. We argue that the evolutionary and ecological experience with PSCs may be required to respond to such toxins. However, this hypothesis must be tested in other systems to determine whether this pattern is a general strategy for coping with PSCs. To better study the role of evolutionary experience in determining responses to PSCs, studies must be conducted to survey clades where herbivory has evolved several times independently, thus allowing for phylogenetic or taxonomic correction [e.g. liolaemid lizards (Espinoza et al., 2004), cyprinid minnows (German et al., 2010) or a wider survey of cricetid rodents (Samuels, 2009)]. Additionally, this question can be investigated at higher trophic levels. For example, insects are able to sequester PSCs into various organs or cuticular structures to then act as anti-predatory agents (Price et al., 1980). Thus, insectivores may come into contact with enzyme-inhibiting toxins, and may employ similar or novel mechanisms to overcome this challenge. These studies, combined with those conducted in insects, will allow better understanding the role of evolutionary history in determining interactions between toxins and the digestive system.

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CHAPTER 3

EFFECTS OF ANATOMY AND DIET ON GASTROINTESTINAL PH IN RODENTS

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RESEARCH ARTICLE

Effects of Anatomy and Diet on Gastrointestinal pH in Rodents

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ABSTRACT

The pH of the gastrointestinal tract can have profound influences on digestive processes. Rodents exhibit wide variation in both stomach morphology and dietary strategies, both of which may influence gut pH. Various rodent species have evolved bilocular (or semi-segmented) stomachs that may allow for more microbial growth compared to unilocular (single-chambered) stomachs. Additionally, herbivory has evolved multiple times in rodents. The high dietary fiber typical of an herbivorous diet is known to induce secretion of bicarbonate in the gut. We predicted that stomach segmentation might facilitate the separation of contents in the proximal chamber from that of the gastric stomach, facilitating a chemical environment suitable to microbial growth. To investigate the effect of stomach anatomy and diet on gut pH, several species of rodent with varying stomach morphology were fed either a high or low-fiber diet for 7 days, and pH of the proximal stomach, gastric stomach, small intestine, and cecum were measured. We discovered that rodents with bilocular stomach anatomy maintained a larger pH gradient between the proximal and gastric stomach compartments, and were able to achieve a lower absolute gastric pH compared to those with unilocular stomachs. Dietary fiber increased the pH of the small intestine, but not in any other gut regions. The stomach pH data supports the century old hypothesis that bilocular stomach anatomy creates an environment in the proximal stomach that is suitable for microbial growth. Additionally, the alkaline small intestinal pH on a high fiber diet may enhance digestion. *J. Exp. Zool. 319A:225–229, 2013.* © 2013 Wiley Periodicals, Inc.

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The gastrointestinal tract is a chemically complex mixture of macromolecules, electrolytes, and enzymes that interact to supply nutrients to the animal. However, certain physicochemical characteristics, such as pH, can alter digestive processes, including the efficiency of digestive enzymes (Cornish-Bowden, '95), nutrient transporters (Thwaites and Anderson, 2007), and microbial fermentation (Erfle et al., '82). Therefore, vertebrates tightly regulate the pH of their gastrointestinal tract through the secretion of HCl from the stomach, and bicarbonate from the pancreas, intestine, and cecum (Schulz, '80; Hopfer and Liedtke, '87; Canfield, '91; Stevens and Hume, '95).

However, variation in gastrointestinal anatomy may alter the pH of gut regions. For example, some species have gastric glands spread through the entirety of the stomach, while others have them reduced to the distal portion (Kararli, '95). Rodents tend to follow the latter condition, yet still exhibit variation in stomach anatomy. Some rodents, such as laboratory mice, exhibit a unilocular stomach, where the stomach exists as a single chamber (Stevens and Hume, '95). Others, such as New World mice (*Peromyscus* spp.), woodrats (*Neotoma* spp.), and voles (*Microtus* spp.) have a bilocular stomach, where a deep invagination near the

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esophageal opening slightly separates two regions of the stomach, with the proximal segment extending above the esophageal opening (Carleton, '73; Stevens and Hume, '95). Although the morphology of the bilocular stomach anatomy in rodents was described over a century ago, its function remains unknown. It has long been proposed that the separation might allow for growth of symbiotic microbes in the proximal chamber (Toepfer, 1891). However, the chemical environments of these chambers have not been investigated in rodents with bilocular stomachs with respect to their suitability for microbial growth. This anatomy may aid in separating the proximal contents of the stomach from the gastric stomach.

Diet is another factor that may influence gastrointestinal pH. Rodents exhibit a wide range of dietary habits, with herbivory having evolved multiple times independently (Samuels, 2009). Dietary fiber increases pancreatic secretion of bicarbonate in a number of mammals, including rodents (Stock-Damge et al., '83; Sommer and Kasper, '84; Zebrowska and Low, '87). Additionally, microbes throughout the gut can produce short-chain fatty acids from easily fermentable carbohydrates, which may locally lower pH (Lupton et al., '88; Yoshioka et al., '94). Thus, diet is likely to alter the gastrointestinal pH of rodents.

Here, we investigated how variation in stomach anatomy and diet might influence gastrointestinal pH. We predicted that species with bilocular stomachs would exhibit different pH values between stomach chambers due to a more enhanced anatomical separation. Additionally we predicted that dietary fiber would increase the pH in the gastrointestinal tract. To test these predictions, we maintained several species of rodents with varying stomach anatomy on both high fiber and low fiber diets, and measured the pH of various gut regions.

MATERIALS AND METHODS

Animals

We conducted diet trials on one species of rodent with unilocular stomachs, the house mouse (*Mus musculus*), and two species with bilocular stomachs [deer mouse (*Peromyscus maniculatus*); desert woodrat (*Neotoma lepida*)] (Carleton, '73). To investigate the effect of diet on gut pH, individuals of all three species were fed either a high fiber diet (Harlan Teklad 2031, Madison, WI, USA), or a low fiber diet (Harlan Teklad 2018), ad libitum for 7 days. Diets are meant to replicate "herbivorous" and "omnivorous" diets, respectively. Though the largest difference between the diets is the content of fiber and easily digestible carbohydrates, they differ in other nutrients as well, namely the low fiber diet contains slightly more protein and fat (Table 1). House mice ($n = 4$/diet) originated from captive, outbred individuals under IACUC #10-07012. Deer mice ($n = 4$/diet) were captive bred individuals under IACUC#11-01007. Desert woodrats ($n = 3$/diet) were collected in nature (Lytle Ranch, Washington Co., UT, USA) and maintained in the laboratory under IACUC #10-01013. All animals used were adults of both sexes. Food intake in this experiment was not measured.

Table 1. Macronutrient composition of experimental diets (% dry matter).

	High fiber[a]	Low fiber[b]
Crude fiber	21.8	3.5
Crude protein	14.8	18.6
Fat	2.3	6.2
Ash	8.3	5.3

[a]Composed primarily of alfalfa, soybean hulls, and oats.[b]Composed primarily of wheat and corn.

We also collected samples from two species without conducting diet treatments. Samples were collected from montane voles (*Microtus montanus*), which have bilocular stomachs (Stevens and Hume, '95) and common spiny mice (*Acomys cahirinus*). Previous reports on the stomach anatomy of a closely related species (*A. spinosissimus*) show varying descriptions (Perrin and Curtis, '80; Boozaier, 2012), and thus we aimed to document the stomach anatomy of *A. cahirinus*. Voles ($n = 3$) were wild-caught individuals, dissected in the field, from Big Creek Canyon, Lander Co., NV, USA, collected under IACUC #09-02004. Traps were baited with just a few seeds and placed on obvious runways of voles. Common spiny mice ($n = 3$) were from breeding colonies at the Department of Biology and Environment at the University of Haifa, Oranim, and fed ad libitum rodent chow (Koffolk 19510, Tel Aviv, Israel) and whole, fleshy fruit of *Ochradenus baccatus*. The experimental protocols were approved by the Committee of Animal Experimentation of the University of Haifa (permit number 096/08).

Individuals of all species were euthanized under CO_2 and immediately dissected. All animals were nocturnal and were dissected within 5 hrs of the beginning of the daylight cycle, and thus had likely completed daily feeding recently. However, voles were an exception as they were dissected directly from traps with limited food, and so may have consumed very little food during the evening. Complete contents of the proximal stomach, distal stomach, small intestine, and cecum were collected, frozen, and transported to the University of Utah. Large intestinal pH was not measured. Gastrointestinal contents were thawed to room temperature, and pH was measured using an Omega Soil pH electrode (PHH-200), which compensates for temperature.

Statistics

For those species in which a diet comparison was conducted (house mouse, deer mouse, and woodrat), we used a repeated measures ANOVA model with species, diet, and gut region as variables. Data were tested for sphericity, and if any violations occurred, Huynd–Feldt corrections were used to compare

treatments. To test whether pH varied by stomach compartment, we conducted post-hoc, paired *t*-tests within each species. Additionally, we tested for diet effects of specific gut region pH values by conducting post-hoc *t*-tests on each gut region, within each species. A Bonferroni corrected value of $\alpha = 0.025$ was used for all post-hoc tests. For species that lacked a diet treatment (spiny mouse, vole), we simply conducted paired *t*-tests between the pH values of stomach regions.

RESULTS

Upon dissection, we learned that *Acomys cahirinus* exhibits bilocular stomach anatomy (Fig. 1).

The data used in the repeated measures ANOVA model violated the assumption of sphericity (Mauchly's Test of Sphericity, $P = 0.047$), and so degrees of freedom were modified by a Huynd–Feldt correction of $= 0.98$ to determine final *P*-values. The gut pH values differed between species, and the pH of contents differed significantly by gut region (Table 2, Fig. 2). The pH of gut regions also varied across species, and gut regions responded differently to diet treatments (Table 2, Fig. 2). Specific pH values for all regions and treatments can be found in Supplementary Table 1.

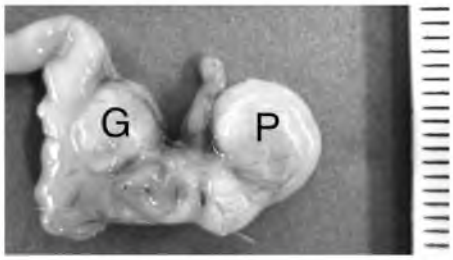

Figure 1. Stomach from a common spiny mouse (*Acomys cahirinus*) showing bilocular anatomy. "P" marks the proximal chamber, "G" marks the gastric chamber. Scale bar shows mm.

Table 2. Statistical results from repeated measures ANOVA of gastrointestinal pH.

Effect	*F*	df	*P*
Species	9.54	2,16	**0.0019**
Diet	2.33	1,16	0.15
Species × diet	1.66	2,16	0.22
Gut region[a]	831.45	2.9,47.3	**<0.0001**
Gut region × species[a]	28.48	5.9,47.3	**<0.0001**
Gut region × diet[a]	3.25	2.9,47.3	**0.031**
Gut region × species × diet[a]	1.71	5.9,47.3	0.13

Significant differences are in bold.
[a]Degrees of freedom have been transformed with Huynd–Feldt correction.

Post-hoc tests investigating regional differences in pH within the stomach revealed the importance of anatomy. Paired *t*-tests for all species with bilocular stomachs (deer mouse, woodrat, vole, and spiny mouse) showed significant differences between the proximal and gastric stomach pH ($P \leq 0.002$ for all species, Figs. 2 and 3). In contrast, the only species with a unilocular stomach (house mouse), showed no differences between proximal and distal stomach pH ($P = 0.58$, Fig. 2).

Post-hoc tests investigating the effect of diet on gut pH revealed that the only region that differed was the small intestine. The high fiber diet significantly increased small intestinal pH in the house mouse ($P = 0.005$) and deer mouse ($P = 0.006$) and showed a trend for increased pH in the woodrat ($P = 0.049$, Fig. 2). All other

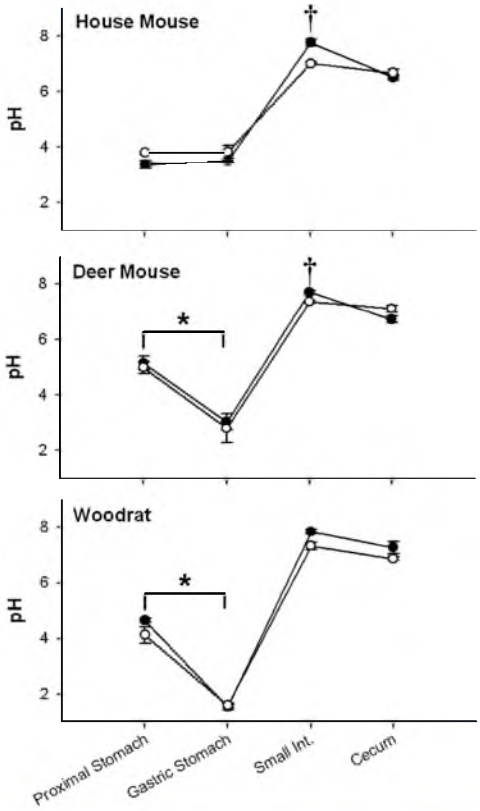

Figure 2. Mean ± SEM pH of gut regions from rodents in diet experiment. $n = 3$–4 per group. Open circles represent low fiber diet, closed circles represent high fiber diet. *Indicates significant differences in pH between the proximal and gastric stomach chambers. †Indicates significant differences between diet treatments in a given gut region.

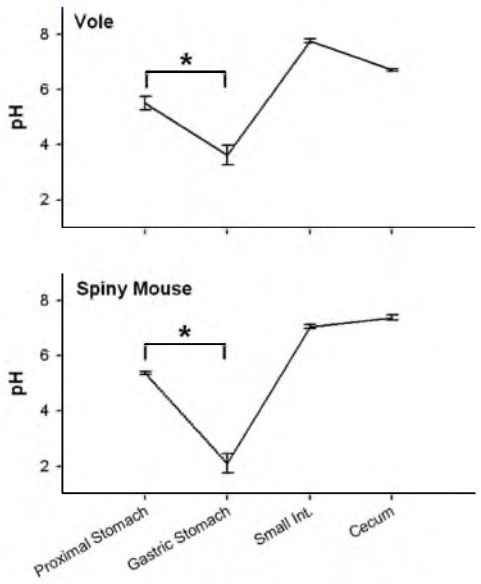

Figure 3. Mean ± SEM pH of gut regions from voles and spiny mice. $n = 3$ for each species. *Indicates significant differences in pH between the proximal and gastric stomach chambers.

Table 3. Comparison of stomach pH between regions from species with bilocular and unilocular stomachs.

	Proximal stomach	Gastric stomach
Bilocular species		
Hamster[a,b]	6.9	2.9
Deer mouse	5.1	2.9
Woodrat	4.4	1.6
Spiny mouse	5.4	2.1
Vole	5.5	3.6
Unilocular species		
Guinea pig[a,c]	4.5	4.1
Rat[a]	5.0	3.3
Mouse[a]	4.5	3.1
Gerbil[a,d]	5.5	3.8
Mouse	3.6	3.7

[a]Stomach pH data from Kararli ('95).
[b]Anatomy assigned based on Carleton ('73).
[c]Anatomy assigned based on Potter et al. ('56).
[d]Anatomy assigned based on Naumova et al. (2011).

gut regions lacked significant differences in pH based on diet treatment.

DISCUSSION

We explored the gastrointestinal pH of rodents with differing stomach anatomy fed both high and low fiber diet treatments. We found that overall gastrointestinal pH differed between species. The results revealed that a bilocular stomach anatomy seems to allow rodents to maintain a pH gradient between stomach chambers. Diet seemed to have little effect on gastrointestinal pH, as we found few differences in pH due to a high fiber diet. The exception was the small intestine, where a high fiber diet caused a more alkaline pH. Below we discuss mechanisms and possible consequences of these findings.

The functional significance of bilocular stomach anatomy in rodents was proposed over a century ago to allow growth of symbiotic microbes in the proximal chamber (Toepfer, 1891). Indeed, we have documented that relatively diverse microbial community resides within this proximal chamber (Kohl and Dearing, 2012). Our study represents the first thorough investigation in to the stomach pH of rodents with bilocular stomachs. Here, we report that this anatomy aids in maintenance of differential pH between chambers. This finding is further supported when the results herein are placed in the context of pH values from previously studied rodents (Table 3). Rodents with bilocular stomachs maintain a larger pH gradient between regions, and achieve an overall lower pH in the gastric region. The only exception is the high gastric pH of the vole, which is likely due to the length of time since feeding in our study. Low gastric pH facilitates digestion of protein and protection against ingested pathogens (Giannella et al., '72; Stevens and Hume, '95), and so bilocular stomachs may function better in these respects compared to unilocular stomachs. These hypotheses, though, remain to be explored in further studies.

We found that when feeding on the high fiber diet, rodents tend to maintain an elevated small intestinal pH. Feeding on a high fiber diet increased the small intestinal pH by 0.35–0.75 pH units, which corresponds to a roughly 2- to 5.5-fold increase in the concentration of hydrogen ions. An increase in small intestinal pH in response to dietary fiber has been documented in cattle (Russell et al., '81). This result is unlikely to be an artifact of the pH of the contents entering the small intestine from the stomach, as no differences in pH based on diet were observed in stomach contents. A larger sample size may have allowed us to detect a difference due to the high fiber diet in other gut regions. Another possible mechanism for lower intestinal pH could be higher microbial production of short-chain fatty acids from easily fermentable carbohydrates in the low fiber diet (Lupton et al., '88; Yoshioka et al., '94). However, this is unlikely to be occurring as the small intestine has the lowest microbial density (Savage, '77), and we did not observe an effect of diet on cecal pH, where the most microbial activity occurs. Differences in rates of coprophagy between animals fed different diets (Franz et al., 2011) or the higher ion binding

capacity of dietary fiber (Eastwood, '92) might also drive this response. With these mechanisms, though, we would expect differences in other gut regions, not only the small intestine. Thus, rodents likely physiologically regulate the luminal intestinal environment at high pH values. Intestinal pH is regulated largely by secretion of bicarbonate (HCO_3^-) by the pancreas and intestine (Schulz, '80; Hopfer and Liedtke, '87). Secretion of bicarbonate is an energy-requiring, active process (Schulz, '80). Therefore, there may be some adaptive significance for the increased intestinal pH exhibited by rodents consuming high fiber diets. Alterations in pH can cause differential ionization of nutrients, enzymes, transporters, and secondary chemicals (Cornish-Bowden, '95; Kararli, '95; Thwaites and Anderson, 2007). It could be that when on a high fiber diet, rodents regulate at a higher pH to yield a more beneficial suite of traits, such as selecting for optimal activity of certain enzymes or transporters. Herbivorous species, given their constant high fiber diet, may have evolved enzymes or transporters that work optimally at this higher pH. However, these hypotheses, like those regarding stomach morphology, remain to be investigated.

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CHAPTER 4

DIVERSITY AND NOVELTY OF THE GUT MICROBIAL COMMUNITY OF AN HERBIVOROUS RODENT (*NEOTOMA BRYANTI*)

Reprinted from Springer and Symbiosis, Vol, 47, K.D. Kohl, R.B. Weiss, C. Dale, and M.D. Dearing "Diversity and novelty of the gut microbial community of an herbivorous rodent (*Neotoma bryanti*)," copyright 2011, with kind permission from Springer Science and Busniess Media.

Diversity and novelty of the gut microbial community of an herbivorous rodent (*Neotoma bryanti*)

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Abstract Mammalian herbivores host diverse microbial communities to aid in fermentation and potentially detoxification of dietary compounds. However, the microbial ecology of herbivorous rodents, especially within the largest superfamily of mammals (Muroidea) has received little attention. We conducted a preliminary inventory of the intestinal microbial community of Bryant's woodrat (*Neotoma bryanti*), an herbivorous Muroidea rodent. We collected woodrat feces, generated 16S rDNA clone libraries, and obtained sequences from 171 clones. Our results demonstrate that the woodrat gut hosts a large number of novel microorganisms, with 96% of the total microbial sequences representing novel species. These include several microbial genera that have previously been implicated in the metabolism of plant toxins. Interestingly, a comparison of the community structure of the woodrat gut with that of other mammals revealed that woodrats have a microbial community more similar to foregut rather than hindgut fermenters. Moreover, their microbial community was different to that of previously studied herbivorous rodents. Therefore, the woodrat gut may represent a useful resource for the identification of novel microbial genes involved in cellulolytic or detoxification processes.

Keywords 16S rDNA · Detoxification · Intestinal microbes · Mammalian herbivore · *Neotoma bryanti*

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1 Introduction

Mammalian herbivores face several challenges when consuming plant material as a primary food source. First, up to 60% of plant biomass may be comprised of indigestible cell wall material such as cellulose, hemicellulose, and lignin, which are refractory to digestion by endogenous mammalian enzymes (Karasov and Martinez del Rio 2007). Additionally, plants produce a wide array of defense chemicals known as plant secondary metabolites (PSMs) to discourage consumption by herbivorous animals (Rosenthal and Berenbaum 1991). These chemicals affect mammalian herbivores through various negative physiological effects such as reducing the efficiency of digestion or altering homeostasis (Dearing et al. 2005).

In order to persist on a poor quality diet, many herbivores maintain a consortium of symbiotic microbes (Van Soest 1994). The primary role of these microbes involves digestion and fermentation of food; the process by which organic polymers such as cellulose are hydrolyzed and converted into short-chain fatty acids that are easily absorbed by the host (Karasov and Martinez del Rio 2007). Additionally, gut microbes are hypothesized to play a role in the detoxification of plant secondary metabolites (Freeland and Janzen 1974). Ideally, detoxification would take place in a pregastric chamber, such that biotransformation of plant secondary metabolites could occur prior to

absorption in the small intestine (Freeland and Janzen 1974). Indeed there are examples of microbial detoxification of PSMs in domesticated ruminants (Jones and Megarrity 1986; Smith 1992; McSweeney and Mackie 1997). However, microbial detoxification of PSMs is only beginning to be investigated in wild mammalian herbivores (Hiura et al. 2010; Sundset et al. 2010)

To date, the most extensive effort to characterize the microbial diversity of the mammalian gastrointestinal tract involved analysis of 16S rDNA sequences from fresh feces of 59 species of non-human mammals (Ley et al. 2008). In this study, dietary strategy, gut morphology, and taxonomic order all strongly influenced the microbial community structure. Within the 33 species of herbivores included in this study, a distinct difference existed in the microbial communities of foregut and hindgut herbivores. Notably, the order Rodentia, which is the most diverse and abundant mammalian order (Musser and Carleton 2005), was underrepresented in this study. Only two herbivorous rodents were sampled, the capybara (*Hydrochoerus hydrochaeris*) and naked-mole rat *(Heterocephalus glaber),* both of which are outside the largest superfamily of mammals, Muroidea (Musser and Carleton 2005).

In this study, we aimed to generate the first inventory of the microbial diversity of an herbivorous rodent species within Muroidea, and to place the data within the context of the other mammalian herbivores inventoried by Ley et al. (2008). To conduct our initial inventory, we chose Bryant's woodrat *(Neotoma bryanti)*. Populations of Bryant's woodrat in the Sonoran desert readily consumes creosote bush (*Larrea tridentata*), which contains high levels of phenolics, a class of PSMs (Hyder et al. 2002), and indigestible material (Meyer and Karasov 1989). To deal with the high fiber content, woodrats, like many rodents, maintain large hindgut fermentation chambers, known as ceca (Fig. 1). Interestingly though, woodrats also have highly segmented stomach morphology (Fig. 1; Carleton 1973). This structure is unique from the capybara and naked-mole rat surveyed by Ley et al. (2008), both of which have simple, uncompartmentalized stomach morphology (Stevens and Hume 2004; Kotze et al. 2010). It has been hypothesized that the segmentation in *N. bryanti* may facilitate the growth microorganisms in the pregastric stomach (see Carleton 1973 for discussion), but this has never been sufficiently tested.

Our goal was to conduct a preliminary inventory of the intestinal microbes of an herbivorous rodent within the superfamily Muroidea, and to compare this diversity with the known microbial community structures of other herbivorous mammals as described in Ley et al. (2008). To accomplish this, we inventoried the microbial community of two randomly chosen individuals of *N. bryanti*. This sample size is on par with the interspecific study of

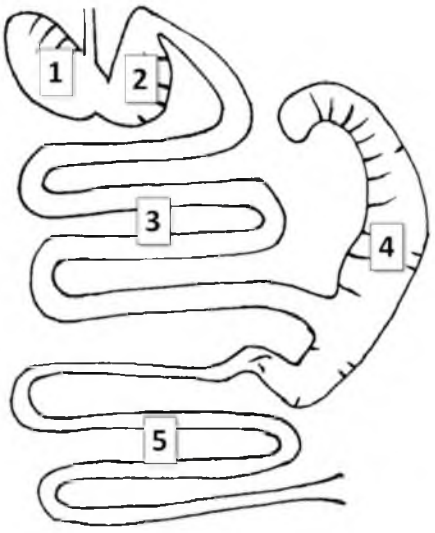

Fig. 1 Diagram of the woodrat gastrointestinal tract. Numbered segments represent 1. Pregastric stomach 2. Gastric stomach 3. Small intestine 4. Cecum 5. Large intestine

microbial diversity conducted by Ley et al. (2008) where most species were represented by a single fecal sample (average=1.6; mode=1 individuals per species of herbivorous mammal). We recognize that a sample size of two individuals does not capture the variability in microbial communities of this species. However, this study was not intended to be a comprehensive intraspecific comparison, but rather to cast the results in an interspecific comparison with a sample size similar to Ley et al. (2008). We hypothesized that *N. bryanti* has a microbial community similar to previously studied hindgut-fermenting rodents. Additionally, based on the represented microbial taxa, we can speculate on the functional significance of the woodrat intestinal microbial community. In addition, we wished to determine the potential for future use of herbivorous rodents in studies investigating microbial detoxification of plant diets. This may be of interest to researchers, as rodents within Muroidea are often small and amenable to laboratory conditions, making them easier to study than captive or wild ruminants.

2 Methods

Animal collection and housing Neotoma bryanti were collected in April 2009, outside Palm Desert, CA (33°68′ N, 116°36′ W) in the Sonoran desert. Woodrats were transported to the University of Utah Department of Biology Animal Facility and housed in individual cages (48×27×20 cm) under a 12:12-hr light:dark cycle, with 28°C ambient

temperature and 20% humidity. Woodrats were maintained on a diet of high-fiber rabbit chow (Harland Teklad formula 2031) for 7 months prior to experimentation. This chow is nutritionally similar to a natural diet consumed by woodrats, but lacks PSMs (Karasov 1989; Meyer and Karasov 1989). The captive conditions experienced by the woodrats are comparable to those of zoo animals studied in Ley et al. (2008). Additionally, Ley et al. (2008) found that host species, rather than environmental effects, largely influences microbial diversity, as evidenced by two baboon individuals (one from Namibia, one from St. Louis Zoo), which had very similar microbial communities. Likewise, two red pandas housed at different zoos had similar microbial communities (Ley et al. 2008). All procedures were approved under University of Utah's Institutional Animal Care and Use Committee protocol number 10–01013.

Dietary treatment Prior to fecal collection for microbial inventories, PSMs from creosote were added to the maintenance diet to better mimic the woodrat's natural diet. We used creosote resin because creosote bush is a common shrub in this habitat; captured woodrats readily consumed creosote clippings added to their cage. Although the actual amount of creosote consumed in the wild was unknown for this population, the diet of *Neotoma lepida*, the sister taxa to *N. bryanti,* consists of greater than 75% creosote bush in the wild (Karasov 1989). We gradually increased the level of creosote resin in the diet over a 10-day period to permit adaptation to the new compounds. Animals were fed a diet consisting of 1% creosote resin for 2 days, 2% for 3 days, 4% for 3 days, and 6% for 2 days. Individuals did not reduce food intake or lose body mass throughout this feeding schedule.

To extract resin, creosote leaves were collected from trapping sites and frozen at −20°C prior to resin extraction. Resin was extracted by soaking leaves in acetone (1:6, wet leaf mass:volume solvent) for 45 min. Solvent and resin were filtered (Whatman filter paper grade 1) to remove large particles and evaporated using a rotovap until the resin was highly viscous, at which point it was transferred to a vacuum pump for 48 h to remove any remaining acetone. Extracted resin was stored at −20°C prior to use.

Creosote diet was prepared by dissolving resin in a volume of acetone equal to 25% of the dry weight of ground rabbit chow to which it was added. Acetone was evaporated in a fume hood, and complete evaporation was confirmed gravimetrically.

Fecal collection We collected feces from two randomly chosen individuals in order to conduct a preliminary microbial inventory with which to compare to other herbivorous mammals. During the final day of feeding, the bedding was completely changed every hour for 5 h. At each changing, feces were collected and immediately placed on ice and later frozen at −80°C.

DNA isolation and sequencing Feces were thawed on ice and several pellets from each individual were ground with a sterilized mortar and pestle. Fecal material (~25 mg) was incubated with 180 μL enzymatic lysis buffer at 37°C for 30 min to degrade the cell walls of gram-positive bacteria. The lysis buffer consisted of 20 mM Tris·Cl, pH 8.0, 2 mM sodium EDTA and 1.2% TritonX-100 dissolved in deionized water, with 20 mg/ml lysozyme added just before use. DNA was then extracted from fecal material using a QIAGEN DNeasy Blood and Tissue Kit. Bacterial 16S rDNA sequences were PCR amplified using universal primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR products were purified using a GeneJET Gel Extraction kit (Fermentas) and cloned using a TOPO TA cloning kit, following the manufacturer's instructions (Invitrogen Corp.). The success of the cloning procedure was validated by restriction enzyme analysis of recombinant plasmid DNA from several clones.

Plasmid DNA was isolated by an automated procedure in the high throughput sequencing facility at the University of Utah Department of Human Genetics. We isolated plasmid DNA from 144 clones per individual with the goal of obtaining a number of high quality sequences that was within the 33–370 sequences per sample obtained in the analysis of herbivorous mammals by Ley et al. (2008). Moreover, each woodrat sampled needed to exceed the 10–40 microbial sequences deemed sufficient for community comparisons (Lozupone et al. 2010). After overnight growth, plasmid DNA was isolated by alkaline lysis, RNase treatment and ethanol precipitation. The cloned 16S rDNA genes were then sequenced using the primer 27F and ABI BigDye Terminator v3.1 cycle sequencing reagents, followed by capillary electrophoresis and detection with an ABI 3730xl DNA analyzer.

Sequence analysis Potential base-calling errors were removed by trimming sequences to remove bases with a PHRED quality score <20. Sequences were aligned to known 16S rDNA sequences using the Near Alignment Space Termination (NAST) algorithm on the GreenGenes website (http://greengenes.lbl.gov) (DeSantis et al. 2006b; DeSantis et al. 2006a). Bellerophon3, with default parameters, was used to identify and remove any chimeric sequences (Huber et al. 2004). Chimera-free sequences were deposited in GenBank and are available under accession numbers HQ700956-HQ701126. Aligned and chimera-free sequences from Ley et al. 2008 were also obtained from the GreenGenes database for comparative analysis. Only sequences from herbivorous mammals were

selected for our interspecific data analysis (n=53 samples from 33 species; Ley et al. 2008), as well as microbial sequences from cattle feces (n=3 samples; Ozutsumi et al. 2005). Although there were other rodents in the complete Ley et al. (2008) data set that are more closely related to woodrats than the naked mole rat or capybara, these species (Prevost's squirrel, rat) were classified by Ley et al. (2008) and others as omnivores (Marshall et al. 2009; Landry 1970). Therefore these two species were not included in the analysis of herbivorous species.

We used NCBI BLAST (Altschul et al. 1997) to determine the percentage of sequence identity between the woodrat gut 16S rDNA sequences and other microbial 16S rDNA sequences in GenBank. It is widely accepted that the cut-offs for sequence identity at the genus and species level are 95% and 97.5%, respectively (Stackebrandt and Goebel 1994; Ludwig et al. 1998). Woodrat microbial sequences were also classified using the Ribosomal Database Project (RDP), with the standard minimum support threshold of 80% (Wang et al. 2007).

To compare the microbial communities of the woodrat with those of herbivorous mammals reported by Ley et al. (2008), we constructed a phylogenetic tree containing all sequences. To decrease the number of sequences used in creating the tree, microbial communities of each mammalian individual were dereplicated using FastGroupII to group sequences with 97% sequence similarity (Yu et al. 2006). A phylogenetic tree was created using FastTree with Gamma20 likelihoods (Price et al. 2010).

Diversity and community structure of mammalian intestinal microbes were determined and compared using Fast UniFrac (Hamady et al. 2010). This program measures phylogenetic beta diversity between environmental samples (in this case, different hosts) with the UniFrac distance metric. UniFrac distances are based on fractions of shared branch lengths between environmental samples using the phylogenetic tree created from all 16S rDNA sequences (Lozupone and Knight 2005). We calculated UniFrac distance metrics between the microbial communities of all herbivorous rodent species (woodrat, capybara, naked-mole rat) and all other herbivorous mammals using unweighted trees (to investigate differences in community membership) and weighted trees (community structure) (Lozupone and Knight 2005). Average distance metrics for each non-rodent species were calculated first so that each mammal species, and not each individual, represented an independent unit. We then compared average distance metrics from rodent species to the communities of foregut vs. hindgut fermenting mammals. These averages were compared using a Student's t-test with JMP 8. To visualize similar communities, UniFrac was used to conduct Principal Coordinates Analysis (PCoA) using an abundance-weighted tree.

3 Results

Microbial diversity of the woodrat gut We obtained a total of 171 high quality, chimera free sequences (77 and 94 per individual) from the feces of *N. bryanti*, with an average sequence length of 1,045 bp. When comparing woodrat individuals to each other, roughly two-thirds of the sequences from each individual were unique at a 97% sequence identity cut-off (Table 1). The microbial phylum Firmicutes was dominant, comprising an average of 94.0±4.6% of sequences for each individual. The remaining sequences belonged to the phylum Bacteroidetes (4.8±4.8%) and the uncultivated phylum TM7 (1.2±0.1%). Approximately half of the sequences were identified at the genus level using RDP (~56%), resulting in nine genera being identified (Table 1). The majority of sequences that could not be identified at the genera level were identified as members of the families Lachnospiraceae and Ruminococcaceae.

BLAST analysis revealed that most sequences (~96%) shared <97.5% sequence identity with their closest relative in the GenBank database, indicating that the majority represented novel species (Fig. 2). Additionally, 38% of sequences shared <95% sequence identity with their closest relative in the GenBank database, indicating that they represented novel genera to the woodrat gut.

Table 1 Identification of 16S rDNA sequences from woodrat feces. Bold taxa represent phyla and italicized represent genera

Animal Taxon	303 % of sequences	310 % of sequences
Firmicutes		
Lactobacillus	24.6	25.6
Ruminococcus	5.2	12.8
Coprococcus	6.5	3.2
Anaerotruncus	1.3	–
Allobaculum	–	20.2
Acetivibrio	–	2.1
Unclassified	61.0	25.5
Bacteroidetes		
Barnesiella	–	3.2
Tannerella	–	1.1
Unclassified	–	5.3
TM7		
TM7 genera incertae sedis	1.3	1.1
Total sequences	77	94
% of sequences unique to sample[a]	63.7	69.7

[a] Sequences were deemed unique if they had <97% sequence identity with any sequences from the other woodrat sample. Inventories from Ley et al. 2008 were not used in this analysis

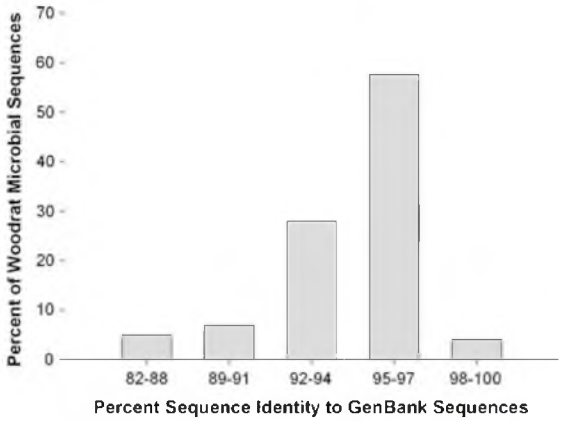

Fig. 2 Distribution of 16S rDNA sequences with varying percent identity to previously reported GenBank sequences. Most sequences represent novel genera or species

Comparison of woodrat microbial diversity with other mammals UniFrac distance metrics were calculated comparing rodent species with all other mammals (Table 2). UniFrac distances comparing Bryant's woodrat and foregut fermenting mammal communities were significantly less than hindgut fermenting mammalian communities, when investigating both community membership and community structure (Students *t*-test: $p=0.034$ and 0.049 respectively; Table 2). UniFrac distances for other rodent species (capybara and naked-mole rat) showed no differences between foregut and hindgut fermenting mammals in terms of community membership or structure. UniFrac distances were larger for Bryant's woodrat compared to other rodent species in all comparisons (Table 2). This is supported by the principle coordinates analysis that shows woodrats cluster far from most mammals, including the other two species of herbivorous rodents (Fig. 3).

Table 2 Mean UniFrac distances from rodent microbial communities to communities of foregut or hindgut fermenting mammals. Values in parentheses represent SE. Sequences for capybara and naked molerat were obtained from Ley et al. 2008. *P*-values in last column represent *t*-test results from horizontal comparisons of UniFrac distances to foregut versus hindgut communities within a given rodent species. Letters following mean values represent Tukey's HSD results from vertical comparisons between rodent species of a given analysis (gut type and non-weighted/weighted distance). Means not sharing the same letter are significantly different at an $\alpha=0.05$

	Foregut unifrac distance	Hindgut unifrac distance	*P*-value
Community membership (unweighted Unifrac distances)			
Bryant's woodrat	0.899 (0.003)^a	0.910 (0.005)^a	0.034*
Capybara	0.812 (0.006)^b	0.805 (0.013)^b	0.664
Naked molerat	0.839 (0.005)^c	0.844 (0.008)^c	0.326
Community structure (weighted Unifrac distances)			
Bryant's woodrat	0.686 (0.014)^a	0.720 (0.014)^a	0.049*
Capybara	0.606 (0.029)^b	0.627 (0.113)^b	0.315
Naked molerat	0.535 (0.021)^b	0.540 (0.025)^c	0.440

4 Discussion

A pervasive interest in the function and ecology of gut microbes has resulted in the cultivation of 10–15% of domestic rumen microbes (Hespell et al. 1997), in comparison to less than 1% of all microbes (Rappe and Giovannoni 2003). There has been a recent call to conduct further research on the microbial ecology of wild ruminants (Kobayashi 2006), and presumably information on other wild herbivores would be beneficial also. In this study we added to the database of Ley et al. (2008) through a preliminary investigation into the intestinal microbial diversity of an herbivorous rodent within the superfamily Muroidea. We found three microbial phyla, and several genera with well studied metabolic processes. Interestingly, Bryant's woodrats were found to harbor a novel microbial community in comparison with other mammals, and this novel community shares more similarity with that of foregut fermenting rather than hindgut fermenting mammals.

We recognize that our microbial community inventories ($n=171$ sequences) originate from two host individuals and from fecal samples. However, the intent of this study was to conduct a preliminary inventory of a new rodent species, and interpret these results in the context of other mammals. The novelty of the microbial species discovered within this species will not be diminished with additional intraspecific sequencing effort. The major aim of this work was to provide data for comparison to a recent interspecific study

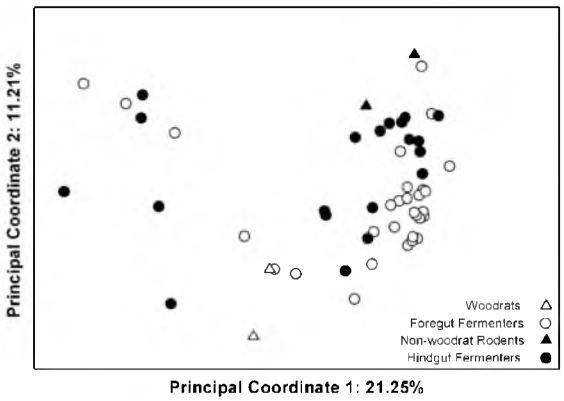

Fig. 3 UniFrac-based Principal Coordinates Analysis of mammalian gut communities using an abundance-weighted phylogenetic tree. All non-woodrat mammal data was obtained from Ley et al. 2008

(Ley et al. 2008). The number of hosts sampled herein was on par with the study conducted by Ley et al. (2008), which sampled the feces, largely from 1 individual per host species of herbivorous mammal. This study was not intended to describe the variability within a species under different conditions or in different regions of the gut.

In our current study, we found that the microbial community of the woodrat gut is comprised mostly of members of the phyla Firmicutes and Bacteroidetes. These are common community representatives in amniote hosts as evidenced by their dominance in the guts of other mammals (Ley et al. 2008), birds (Lu et al. 2003), and reptiles (Costello et al. 2010). The woodrat gut also contains members of the phylum TM7, which has not yet been culitvated in a laboratory setting. TM7 is also present in the guts of some other mammals, comprising between 0% and 2% of all 16 rDNA sequences (Ley et al. 2008).

The known metabolic properties of genera identified in our study provide insights into the putative function of the woodrat gut microbial community. For example, the predominant genus was *Lactobacillus,* a genus known to ferment a wide range of simple sugars. However, *Lactobacillus* does not ferment plant fiber, as it is unable to utilize complex polysaccharides such as cellulose (Barrangou et al. 2006). We also identified the presence of *Ruminococcus*, a genus known to degrade plant polysaccharides into short-chain fatty acids (Forsberg et al. 1997).

Identified genera in the woodrat gut may also play a role in the metabolism of plant secondary compounds. For example, isolates of the genus *Coprococcus* are able to catabolize and utilize phloroglucinol, a phenolic, as a sole carbon substrate (Patel et al. 1981). Also, although the majority of the metabolic properties of TM7 are unknown, members of this phylum are known to be able to degrade toluene, a common aromatic hydrocarbon (Luo et al. 2009). Additionally, members of *Lactobacillus* are able to degrade plant polyphenolics, and seem to be essential for the ability of the Japanese wood mouse (*Apodemus speciosus*) to feed on polyphenolic-rich acorns (Shimada et al. 2006). It is believed that the microbial enzymes involved in aromatic hydrocarbon degradation may have broad substrate specificity (Alvarez and Vogel 1991; Bauer and Capone 1988), and thus members of *Coprococcus,* TM7, and *Lactobacillus* present in the woodrat gut might play a role in degrading the polyphenolics present in creosote leaves. However, further investigations such as metagenomic sequencing, culture of phenolic-degrading microbes, and comparisons of microbial communities across diet treatments are required to rigorously establish whether these microbes biotransform dietary toxins.

The metabolic functions of many of the microbes present in the woodrat gut, though, cannot even be inferred due to the high amount of novelty. According to percent sequence identity taxonomic classification, roughly 38% of the sequences represent novel genera that have not previously been isolated from any environmental sample. This result is noteworthy given the large amount of sequences of mouse (*Mus musculus*) and rat (*Rattus norvegicus*) intestinal microbes deposited in GenBank from various studies (Rawls et al. 2006; Brooks et al. 2003). It is interesting that *N. bryanti* is in the same superfamily as these laboratory rodents, yet does not seem to share microbial species with them. The novel microbes in the woodrat gut may represent a unique community for the mining of novel genes associated with detoxification and cellulolytic processes. For example, a recent metagenomic inventory of the Tammar wallaby (*Macropus eugenii*) foregut uncovered deeply divergent bacterial lineages, as well as microbial polysaccharide utilization genes unique to those found in termites or bovine rumina (Pope et al. 2010).

Comparisons between the gut microbial communities of woodrats and those of other mammals revealed that the woodrat microbial community shares more similarity with that of foregut fermenting mammals in terms of both community membership and structure. Indeed, Fig. 2 shows the microbial community of one woodrat individual superimposed over that of the red river hog (*Potamochoerus porcus)*. Moreover, woodrats cluster closely with the babirusa (*Babyrousa babyrussa)* and springbok *(Antidorcas marsupialis).* Finally, woodrats have a microbial community unlike that of the other herbivorous rodents sampled. These results are surprising given that woodrats have a fermentative cecum in their hindgut (Skopec et al. 2008). It has been suggested that differences between the microbial communities of hindgut and foregut fermenting mammals are a result of the fact that the fermentative microbes of foregut fermenting mammals are emptied into the gastric stomach and digested (Ley et al. 2008). Therefore, future investigations into the microbial community of the woodrat pregastric stomach are warranted. Additionally, further mammalian microbial inventories may illustrate whether this microbial community structure is typical of other herbivorous rodents with segmented stomachs (e.g. voles [*Microtus spp.*]; Stevens and Hume 2004)

Our goal was to conduct a preliminary inventory of the microbial diversity of the woodrat gastrointestinal tract. Interestingly, this study shows that the gut microbial community of herbivorous woodrats is novel, and more similar to other foregut fermenting mammals. It is believed that the number of genes encoded by the gut microbial community outnumbers that of the host by 100-fold (Ley et al. 2006), and thus the woodrat gut may represent a unique community for the identification of novel genes associated with detoxification and cellulolytic processes. Additionally, the fact that woodrats have a community unlike that of other rodents highlights the importance for investigating the

microbial communities of other mammals with unique dietary preferences or digestive physiologies. Woodrats hold promise for a tractable system in which to investigate the function of gut microbial communities under different environmental conditions such as across the regions of the gut or under different dietary treatments.

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CHAPTER 5

WILD-CAUGHT RODENTS RETAIN A MAJORITY OF THEIR NATURAL GUT MICROBIOTA UPON ENTRANCE INTO CAPTIVITY

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Wild-caught rodents retain a majority of their natural gut microbiota upon entrance into captivity

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Summary

Experiments conducted on captive animals allow scientists to control many variables; however, these settings are highly unnatural. Previous research has documented a large difference in microbial communities between wild animals and captive-bred individuals. However, wild-caught animals brought into captivity might retain their natural microbiota and thus provide a better study system in which to investigate the ecology of the gut microbiome. We collected individuals of the desert woodrat (*Neotoma lepida*) from nature and investigated changes in the microbial community over 6 months in captivity. Additionally, we inventoried potential environmental sources of microbes (food, bedding) from the wild and captivity. We found that environmental sources do not make large contributions to the woodrat gut microbial community. We documented a slight decrease in several biodiversity metrics over 6 months in captivity, yet the magnitude of change was small compared with other studies. Wild and captive animals shared 64% of their microbial species, almost twice that observed in other studies of wild and captive-bred individuals (≤ 37% shared). We conclude that wild-caught animals brought into captivity retain a substantial proportion of their natural microbiota and represent an acceptable system in which to study the gut microbiome.

Introduction

The gut microbiome and its role in the ecology and evolution of animals is a burgeoning area of interest (McFall-Ngai *et al.*, 2013). For ease of study, most comparative and experimental studies regarding the microbiota house animals in captive settings (Ley *et al.*, 2008; Kohl and Dearing, 2012). However, it is possible that captivity may alter the microbiota. Indeed, many studies have compared wild and captive individuals and found significant differences in microbial community composition (Uenishi *et al.*, 2007; Ley *et al.*, 2008; Scupham *et al.*, 2008; Villers *et al.*, 2008; Xenoulis *et al.*, 2010; Wienemann *et al.*, 2011; Nelson *et al.*, 2013). However, all these studies compared animals born in captivity with animals born in the wild, with some individuals living on different continents. Thus, these studies cannot exclude the possibility that differences were the result of unique microbial sources. Only a single study, conducted on Atlantic cod, documented a decrease in microbial diversity as animals entered captivity (Dhanasiri *et al.*, 2011). Such as study has not been conducted on a wild tetrapod species.

The desert woodrat, *Neotoma lepida*, is a model system to study interactions between dietary plant toxins and the gut microbiota. We previously demonstrated that plant toxins significantly alter microbial community structure and diversity of woodrats in captivity (Kohl and Dearing, 2012). However, the effect of captivity on these microbial communities was not studied. To address this deficiency in our understanding of the system, we conducted a study to examine the effects of captivity. Several *N. lepida* were collected from the Mojave desert in an area dominated by creosote bush (*Larrea tridentata*), black brush (*Coleogyne ramosissima*) and rabbit brush (*Chrysothamnus paniculatus*). Because woodrats are herbivorous and known to feed primarily on creosote bush (Karasov, 1989), these plants may serve as environmental sources of microbes. In captivity, woodrats were fed commercial rabbit chow (Harlan Teklad 2031) and kept in plastic cages with wood shavings as bedding material. While our study does not isolate the effects of captivity and dietary changes, the use of commercial diets is common, and so this design is of more relevance to comparative biologists.

We investigated environmental sources of microbes in wild and captive settings by inventorying the microbial communities from three dominant plant species in the wild, commercial rabbit chow and wood shavings, and then comparing these with woodrat fecal microbial communities. Additionally, we monitored changes in microbial diversity by inventorying the fecal microbial communities of four *N. lepida* in the wild and over three time points in captivity (2 weeks, 3 months and 6 months).

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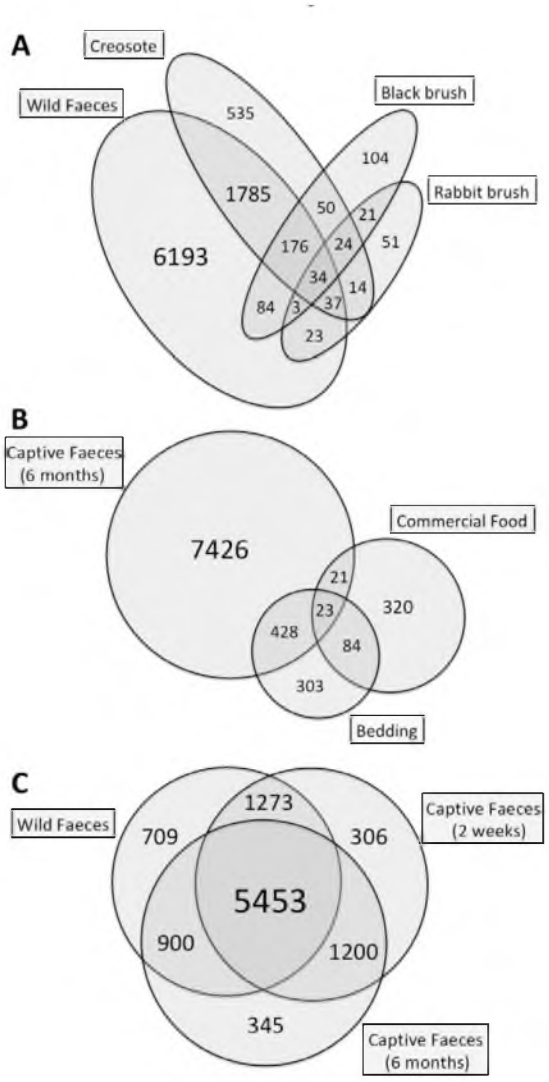

Fig. 1. Shared microbial OTUs between (A) faeces collected from the wild and the surfaces of three dominant plants from where animals were collected; (B) faeces collected after 6 months in captivity and commercial food and bedding; and (C) faeces collected from the wild and after 2 weeks and 6 months in captivity.

Results and discussion

Microbial inventories were conducted by isolating microbial DNA from plant surfaces, food, bedding and faeces, and amplifying and sequencing the 16S rRNA gene on an Illumina MiSeq platform (Caporaso *et al.*, 2012). Over 2 million microbial sequences were produced, which were classified into operational taxonomic units (OTUs) based on 97% sequence identity using QIIME (Quantitative Insights Into Microbial Ecology; Caporaso *et al.*, 2010). Sequences were deposited in the National Center for Biotechnology Information's Sequence Read Archive under accession SRP029350. Details regarding animal collection, sequencing and data analysis can be found in the Supplementary Material.

The gut microbial communities of woodrats in nature were not solely determined by environmental sources. Only ~25% of OTUs detected in the faeces of wild woodrats were also detected on leaf surfaces (Fig. 1A). The largest proportion of these microbes were also present on creosote bush, which is the predominant species consumed by *N. lepida*. After 6 months in captivity, there was minimal inoculation by new environmental microbes. Only 6% of OTUs detected in the faeces of captive woodrats were also detected on commercial food and bedding (Fig. 1B). These results are consistent with a number of studies suggesting that diet and other environmental sources are not the main determinants of the gut microbiome. As examples, less than 1% of the gut microbes found in the Burmese python gut are derived from a rodent meal (Costello *et al.*, 2010), and roughly 3% of seal gut microbes are acquired from sea water (Nelson *et al.*, 2013).

We also compared microbial OTUs between faeces collected in the wild and along three time points up to 6 months in captivity. Woodrats lost 19% of their natural microbes after 2 weeks in captivity (1609 of 8335) and 24% after 6 months (1982 of 8335; Fig. 1C). This effect may have been due to the removal of the natural diet of creosote bush. However, of this proportion of microbes lost in captivity, only a quarter of them were also detected on environmental sources (wild plants). Thus, these lost microbes might represent transient microbes that originate from other environmental sources in the wild that we did not inventory (other plants, soil, etc.) or microbes that were lost because of changes in host physiology in captivity. After 6 months in captivity, woodrats harboured 1545 new OTUs that were not detected in the wild, although only 38 of these were detected on commercial food or bedding. These newly acquired microbes may have come from other sources that we did not inventory (researchers, water, etc.) or they may have been resident in the wild microbiota, and only increased to detectable levels after 6 months in captivity.

Overall, 68% of microbial OTUs were present both in fecal samples from the wild and after 2 weeks in captivity (6726 of 9841), and decreased to 64% after 6 months in captivity (6353 of 9880; Fig. 1C). This overlap is much greater than that observed in previous studies comparing animals born in captivity to animals born in the wild. Both captive leopard seals and parrots only share 4% of OTUs with their wild counterparts (Xenoulis *et al.*, 2010; Nelson *et al.*, 2013). Likewise, wild and captive turkeys only share 37% of their microbes (Scupham *et al.*, 2008). Our study demonstrates that wild-caught animals maintain the

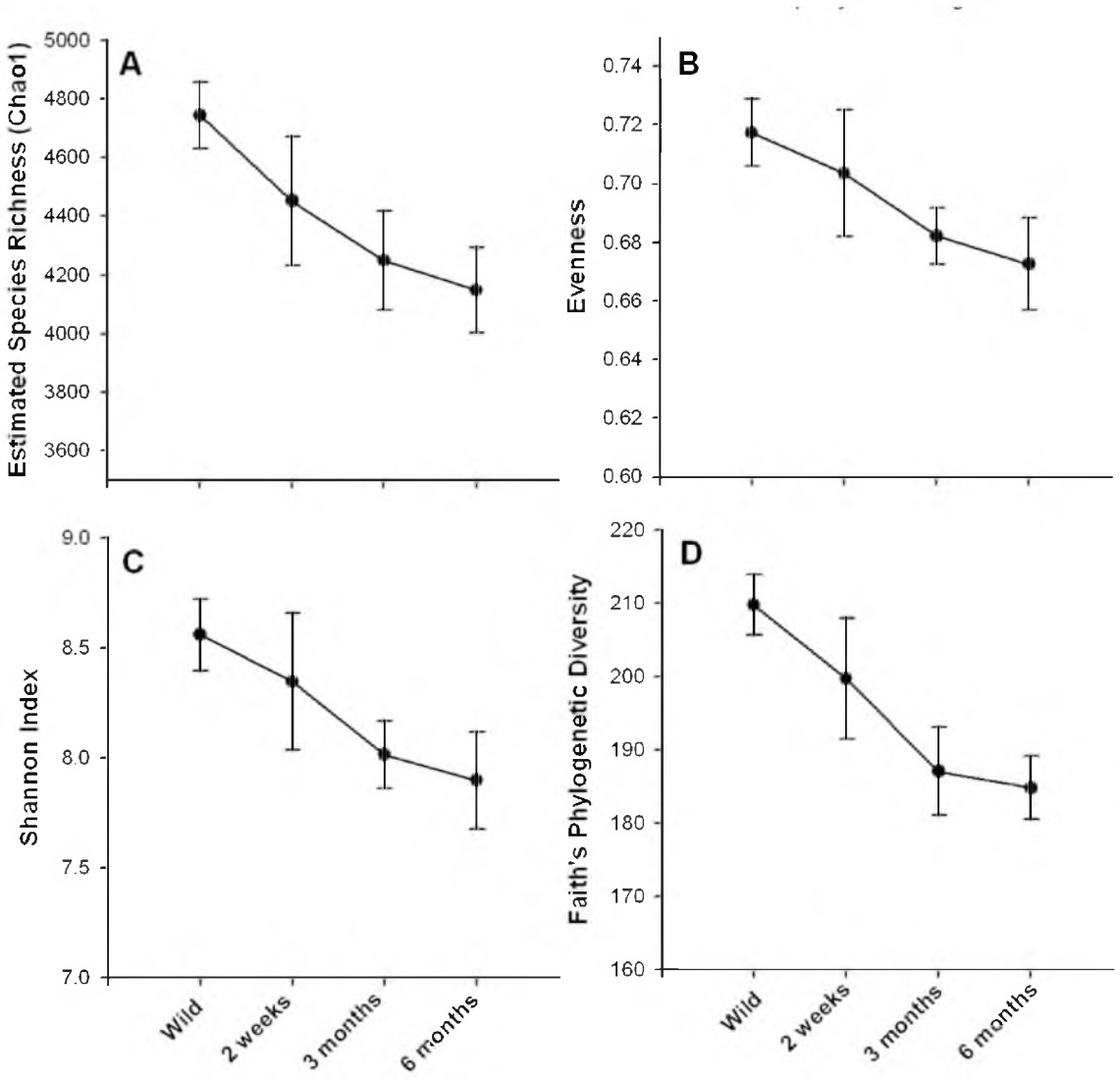

Fig. 2. Measurements of alpha diversity over time in captivity. (A) Estimated species richness (Chao1); (B) Evenness; (C) Shannon Index; (D) Faith's phylogenetic diversity.

majority of their native microbiota after being in captivity for a substantial period of time.

The gut microbial community exhibited a minimal change in biodiversity as animals entered captivity. We monitored changes in several metrics of biodiversity, such as estimated species richness, evenness, Shannon index and Faith's phylogenetic diversity index (Fig. 2). Across all biodiversity measures, only evenness decreased significantly over time in captivity (repeated measures analysis of variance: $P = 0.03$; Fig. 2B). This loss in biodiversity was small relative to previously documented changes. For example, the addition of the plant toxins of creosote bush to the diet can alter microbial diversity by 20–30% in *N. lepida* (Kohl and Dearing, 2012). For comparison, 6 months in captivity woodrats resulted in only a 6–12% decrease in biodiversity depending on the metric.

We also investigated how captivity altered overall community membership (the presence and absence of certain microbes) and community structure (their relative abundances). We conducted principal coordinates analysis of unweighted or weighted UniFrac data to investigate changes in community membership and structure, respectively, and compared clustering based on either individual animal or time in captivity using the adonis

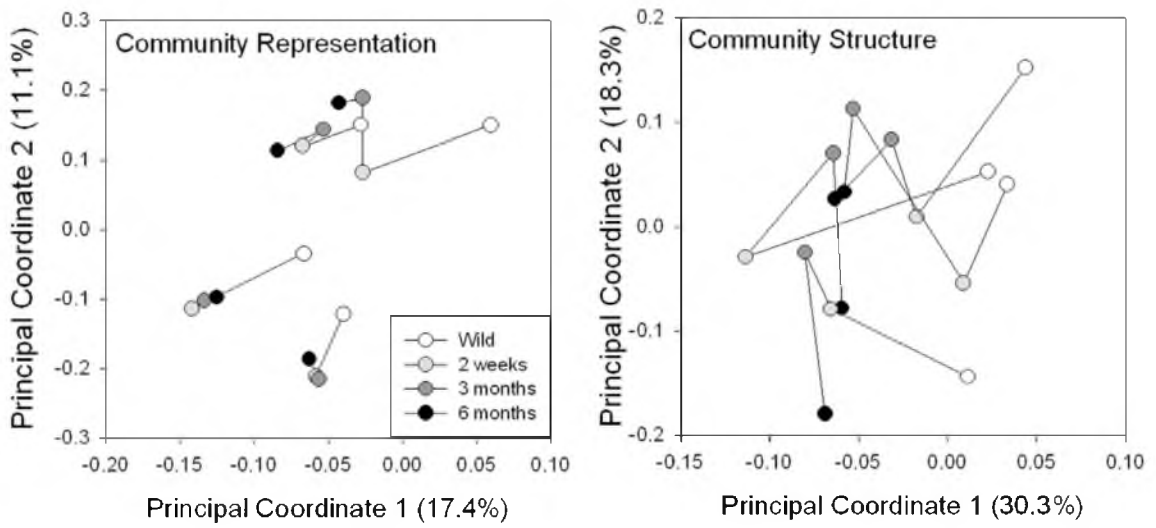

Fig. 3. The effect of captivity on (A) micriobial community representation, and (B) microbial community structure. Community representation uses unweighted UniFrac distances, while structure uses weighted distances. Lines connect an individual animal over time.

function within QIIME. Microbial community memberships clustered by individual animal ($P = 0.006$; Fig. 3A) but not by length of time in captivity ($P = 0.3$). In contrast, community structures clustered significantly according to time in captivity ($P = 0.008$; Fig. 3B) but not by individual animal ($P = 0.2$). These data suggest that as animals enter captivity, they retained the unique microbial community harboured in the wild, while the relative abundances of the communities shifted over time. These changes in community structures are most likely driven by the change in diet that woodrats experience upon entering captivity (Turnbaugh *et al.*, 2009). Also, captivity is known to affect many physiological traits that might influence microbial community structure, such as immune function (Martin *et al.*, 2011), stress physiology (Dickens *et al.*, 2009) and gut anatomy (Millan *et al.*, 2001).

The shifts in community structure were driven by changes in the relative abundances of certain microbial taxa. We conducted paired *t*-tests on the abundances of microbial taxonomic groups between faeces collected in the wild and after 6 months in captivity, and corrected *P*-values using the false discovery rate control. While there were no significant changes in the abundances of any identified microbial taxa, there were several near-significant trends. There was a decrease, although not significant, in the abundance of the phylum *Tenericutes* after 6 months in captivity ($P = 0.09$), from 1.6% of the total community in the wild to roughly 0.3% in captivity. At the genus level, the *Ruminococcus* (wild: $7.4 \pm 2.9\%$; captive $15.9 \pm 2.6\%$; $P = 0.08$) and *Coprococcus* (wild: $0.7 \pm 0.4\%$; captive: $1.4 \pm 0.3\%$; $P = 0.06$) slightly increased, whereas *Adlercreutzia* (wild: $0.15 \pm 0.03\%$; captive: $0.02 \pm 0.01\%$; $P = 0.06$) slightly decreased. There were significant changes in abundance of many unidentified microbes that drove the changes seen in overall community structure.

Studying animals in captivity offers the obvious benefit of being able to isolate a few variables of interest and their effects on the study organism. The main downfall is that laboratory conditions are often unnatural. These advantages and disadvantages also pertain to the study of gut microbial communities, where these communities may experience loss or gain of microbes. Overall, we found that bringing *N. lepida* into captivity from the wild significantly altered the microbiota. However, these changes were smaller than predicted based on previous studies that compared animals born in captivity with animals born in the wild. We documented a markedly higher overlap in microbial OTUs between wild and captive samples than previous studies. Additionally, changes in biodiversity over 6 months in captivity were relatively small compared with changes seen as the result of small dietary changes. We conclude that the use of wild-caught individuals in gut microbial studies is acceptable for short periods of time in captivity, while subsequent studies should monitor microbial diversity over longer periods of captivity. Additionally, future studies should investigate changes in microbial gene expression caused by captivity, which may exhibit larger changes and greatly impact host physiology.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supplementary methods.

CHAPTER 6

HERBIVOROUS RODENTS (*NEOTOMA SPP.*) HARBOUR ABUNDANT AND ACTIVE FOREGUT MICROBIOTA

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Herbivorous rodents (*Neotoma spp.*) harbour abundant and active foregut microbiota

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Summary

Symbiotic gut microbes have facilitated the success of herbivorous mammals, which are generally grouped into foregut- and hindgut-fermenters. However, rodents are primarily herbivorous and exhibit a variety of gastrointestinal anatomies. Most rodents house microbes in hindgut chambers, such as the caecum and colon. Some rodents also exhibit stomach segmentation with a foregut chamber proximal to the stomach. For over a century, scientists have hypothesized that this foregut chamber houses a microbial community, yet this has never been explicitly examined. We investigated the capacity of each of the gut regions to house microbes by measuring size, pH, bacterial cell density, concentrations of microbial metabolites and digesta transit time in woodrats (*Neotoma spp.*). We also compared microbial communities across gut chambers, as well as faeces, using 16S rRNA sequencing. This allowed us to test the appropriateness of using faeces as a proxy for microbial communities of other gut chambers. We found that woodrats house foregut microbial communities with similar density and volatile fatty acid concentrations to rumen ecosystems. Resident microbial communities varied between gut chambers, and faecal bacterial communities were significantly different from caecal and colonic communities. The foregut microbiota may provide a number of physiological services to the host.

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Introduction

Mammalian herbivores have repeatedly evolved symbiotic relationships with gut microbes that contribute significantly to the digestion of fibre (Stevens and Hume, 2004). These gut microbes may reside in stomach chambers, such as in ruminants, macropod marsupials and some primates, or in distal gut chambers, such as in equids, elephants and rabbits (Stevens and Hume, 2004). The location of these gut chambers can have a profound influence on gut microbial communities such that foregut- and hindgut-fermenting mammals harbour unique communities (Ley *et al.*, 2008). Most gut microbial ecology studies focus on ruminants and other large-bodied herbivores (Pope *et al.*, 2010; Hess *et al.*, 2011; Zhu *et al.*, 2011). Rodents are generally herbivorous and form the most diverse and abundant mammalian order (Stevens and Hume, 2004). However, their gut microbial ecology remains understudied compared with other groups.

Rodents are an especially interesting group to study gut microbial communities, as they exhibit a wide variation in gut anatomy. Most rodents house microbes in a caecum in the hindgut; however, some also exhibit segmentation of the stomach and have a foregut chamber. It has been proposed for over a century that the rodent foregut houses a microbial community, presumably for fibre digestion (Toepfer, 1891; Carleton, 1973). Woodrats (*Neotoma spp.*) exhibit foregut segmentation (Kohl *et al.*, 2011) and represent an ideal system for studying adaptations to herbivory (Dearing *et al.*, 2000). The idea of a foregut microbial community is supported by a slightly elevated pH of ~4.5 (Kohl *et al.*, 2013) and documentation of a diverse microbial community in the woodrat foregut (Kohl and Dearing, 2012). Additionally, the microbial communities of woodrat faeces more closely resemble those of foregut- rather than hindgut-fermenting mammals (Kohl *et al.*, 2011). However, the functional nature of the rodent foregut has not been determined.

This unique gut chamber is likely to harbour a novel microbial community compared with other gut regions. Previous studies have documented that microbial communities change along the length of the gut. Mice colonized with eight bacterial species show different relative and absolute abundances of microbes between gut regions (Sarma-Rupavtarm *et al.*, 2004). Likewise, the

Table 1. Mean ± 1 SEM pH and relative masses (percent of body mass) of luminal contents of various regions of the gut of *N. albigula*.

Region	Percent of body mass	pH
Foregut	1.88 ± 0.28	4.40 ± 0.14
Stomach	2.07 ± 0.18	1.37 ± 0.09
Small intestine	2.42 ± 0.48	6.98 ± 0.15
Caecum	6.13 ± 0.32	6.33 ± 0.07
Large intestine	2.05 ± 0.25	6.42 ± 0.11
Faeces	–	6.37 ± 0.16

human large intestine exhibits greater microbial diversity than the small intestine (Wang *et al.*, 2005). These differences are the result of several factors, such as changes in nutrient concentration, pH and flow rate (Harrison *et al.*, 1975; Palframan *et al.*, 2002). However, studies comparing gut microbial communities across gut regions, as well as validating the use of faeces as a representation of various gut regions, have not been conducted.

We conducted a thorough investigation into the capacity of various gut regions of woodrats to house microbial communities by measuring the size, pH, bacterial cell density, concentrations of microbial metabolites and digesta transit time. Additionally, we compared bacterial diversity among gut regions by conducting bacterial inventories with 16S rRNA sequencing. This study also allowed us to investigate the appropriateness of using faeces as a proxy for the communities residing in other gut chambers.

Results

Gut regions varied significantly in their capacity to house microbial communities. The relative mass of luminal contents varied across regions, with the caecum being the largest chamber (Table 1). The pH of various gut regions also differed, with the gastric stomach having an extremely low pH (1.4), the foregut having a moderately low pH (4.4) and the rest of the gut being near neutral (Table 1). The majority of microbial cells in the gastrointestinal tract were dead or injured, as shown by flow cytometry (Supporting Information Fig. S1). However, the gut still harboured a dense community of live microbial cells (Fig. 1). The woodrat foregut harboured a microbial community with a density of live cells on par with that of other, well-known microbial chambers, such as the caecum and large intestine (Fig. 1).

Measurements of microbial metabolites revealed that the foregut microbial community is quite active. Concentrations of acetate, valerate, total volatile fatty acids (VFAs) and NH_3-N were all higher in the foregut chamber, whereas concentrations of butyrate were higher in the caecum (Table 2).

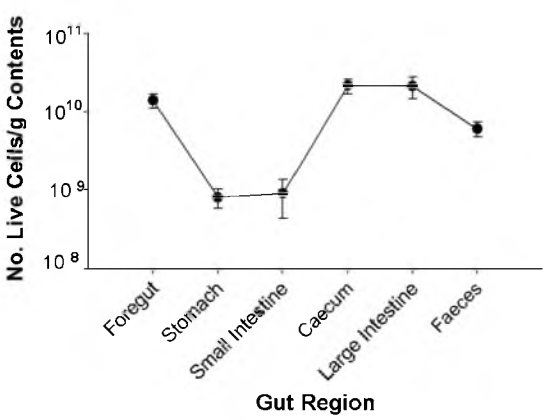

Fig. 1. The density of live microbial cells in each gut region.

Digesta passed through the woodrat gut relatively quickly. The last meal (food eaten < 1.5 h before dissection) generally filled the foregut and reached the stomach and small intestine in this time (Fig. 2). Only 13 ± 4% of foregut contents were from food consumed more than 1.5 h before dissection, and no food eaten more than 3 h before dissection was found in this chamber. Meals progressed through the anterior gut relatively quickly and were then retained in the hindgut for several hours, such that food eaten between 3 h and 7.5 h before dissection all tended to be found in the large intestine (Fig. 2).

Sequencing effort resulted in 619 625 high-quality sequences (average of 20 654 ± 715 sequences per sample. These sequences were assigned to 15 799 operational taxonomic units (OTUs) at 97% sequence similarity. We were able to accurately assign 83.4% of OTUs to bacterial phyla and only 7.7% of OTUs to microbial genera. Relative abundances of 5 of the 12 most dominant phyla showed significant differences between gut regions (Fig. 3A, Table 3). Firmicutes was the dominant phylum in the stomach, caecum and large intestine, while Bacteroidetes comprised the majority of the community in the foregut, small intestine and faeces. Diversity

Table 2. Metabolite concentrations of volatile fatty acids and ammonia nitrogen (mM) in the foregut and caecum of *N. albigula*.

Metabolite	Foregut	Caecum	*P* value
Acetate	164.9 ± 4.6	109 ± 10.1	**0.01**
Propionate	15.5 ± 2.2	12.7 ± 0.5	0.31
Butyrate	12.1 ± 2.2	21.5 ± 4.0	**0.04**
Isobutyrate	2.0 ± 1.4	0.3 ± 0.1	0.31
Valerate	0.3 ± 0.1	0.5 ± 0.1	**0.04**
Isovalerate	0.4 ± 0.1	0.3 ± 0.1	0.25
Total VFA	195.2 ± 4.6	145.1 ± 11.5	**0.02**
NH_3-N	43.5 ± 5.0	20.6 ± 2.6	**0.03**

Concentrations were compared between these chambers with a paired *t* test. Significant differences are in bold.

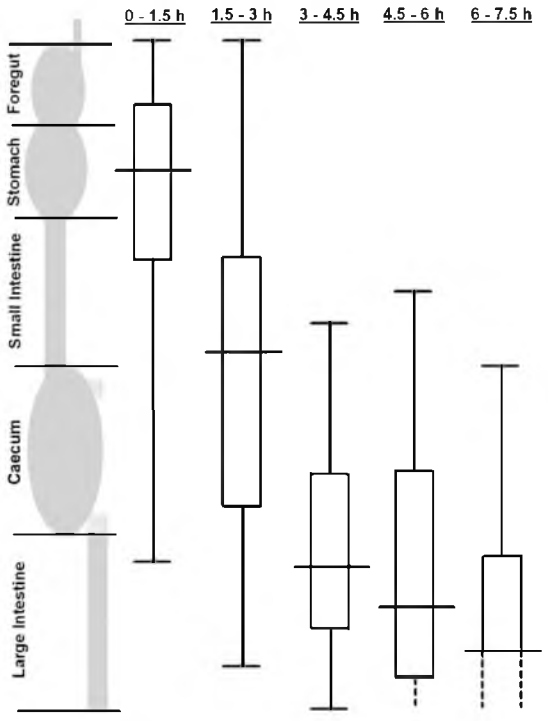

Fig. 2. Digesta movement through the gut of *N. lepida*. Box plots showing the median, quartile, minimum and maximum locations of food eaten at different time points. Dashed lines mean that the measurement includes faecal material. Sizes of gut compartments are not to scale.

also varied at the genus level, with relative abundances of four of the five most dominant identified genera exhibiting significant differences between gut regions ($P \leq 0.01$ for *Oscillospira*, *Lactobacillus*, *Desulfovibrio* and *Ruminococcus*; Fig. 3B). It is also noteworthy that we were only able to accurately identify less than 25% of sequences in any gut region to the genus level. The 'Other' category of genera (Fig. 3B) represents sequences that were identified to the genus level, but are present at very low abundances. This category contains 57 identified genera that collectively make up less than 8% of the community of any region. A large proportion (> 75%) of the community was unable to be identified to the genus level (termed 'Unidentified'), and contained 14 578 OTUs when grouped at 97% sequence identity. Biodiversity metrics (Shannon index, estimated species richness, evenness and phylogenetic diversity) also varied significantly by gut region ($P < 0.001$ for all metrics, Fig. 4), with the small intestine consistently showing the lowest diversity.

Both the individual animal and gut region source of samples influenced bacterial community membership and structure. Community membership, or the presence and absence of bacterial lineages, was primarily driven by gut region (adonis: $R^2 = 0.28$; $P = < 0.001$; Fig. 5) and also by the individual animal ($R^2 = 0.05$; $P = 0.02$). Community structure, which takes relative abundances of taxa into account, was only driven by gut region ($R^2 = 0.55$; $P = < 0.001$) and not by individual animal ($R^2 = 0.04$; $P = 0.27$). These data suggest that gut region determines bacterial community membership and structure, and that individuals have signature bacterial communities memberships across these regions. This is further supported by the principal coordinates analysis (PCoA) results, where samples with similar communities cluster together on the graph. Gut regions parse out based on principal coordinate 1 (15.4% and 54.5% variation explained for membership and structure, respectively), whereas individual animals parse out on principal coordinate 2 (Fig. 5).

Comparisons between gut regions reveal that adjacent chambers rarely share the most similar communities. Similarities between adjacent communities were observed only once, where the bacterial community membership was most similar between the caecum and large intestine (Fig. 6). Rather, disparate regions share similar communities, as shown by the UPGMA trees (Unweighted Pair Group Method with Arithmetic Mean) of both community membership and community structure (Fig. 6). For example, faeces are the most similar to the foregut in terms of community membership and most similar to the small intestine in terms of community structure (Fig. 6).

Discussion

For over a century, scientists have speculated about the microbial dynamics of the rodent foregut. To our knowledge, this study represents the most thorough investigation into the microbial communities and activity of this chamber, as well as elsewhere in the gut. We found that woodrats maintain diverse and dynamic gut communities across the length of their gastrointestinal tracts and that both the gut region as well as individual animal determine these communities.

The woodrat gut varied across regions in its capacity to house microbes. The caecum was the largest chamber by volume, comprising roughly 6% of the animal's body mass. This size is similar to other herbivorous rodents, such as naked mole rats, porcupines and capybara (Stevens and Hume, 2004). The woodrat foregut was smaller than the caecum and made up less than 2% of the animal's body weight. The foregut was comparatively smaller than the foregut chambers in other animals, such as ruminants and kangaroos, which comprise ~10% of the animals' body mass (Stevens and Hume, 2004). Despite its small size, the woodrat foregut houses microbes at a density similar to that of other well-known microbial communities, such as the caecum and large intestine.

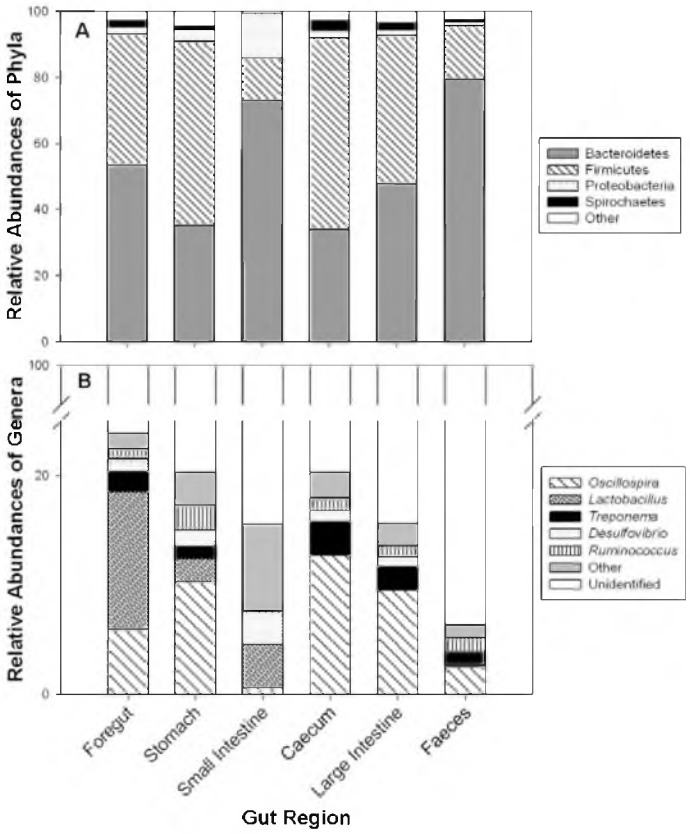

Fig. 3. Relative abundances of dominant bacterial (A) phyla and (B) genera in the gut of *N. albigula*. Rare genera that were assigned with confidence were grouped together.

Table 3. Statistics from ANOVA testing differences of relative abundances of dominant bacterial phyla between gut chambers.

Phylum	*P*
Bacteriodetes	**< 0.001**
Firmicutes	**< 0.001**
Proteobacteria	NS
Spirochaetes	NS
Tenericutes	**0.007**
Cyanobacteria	**0.015**
Elusimicrobia	NS
Deferribacteres	NS
Actinobacteria	**0.008**
Verrucomicrobia	NS
Fusobacteria	NS
TM7	NS

P values have been corrected using the false discovery rate control. Significant differences are in bold.

Moreover, the microbial density of the foregut was similar to the cow rumen (Stevens and Hume, 2004).

Despite similar cell densities, the concentrations of several isolated VFAs, as well as total VFAs, were higher in the foregut chamber than in the caecum. These metabolites are produced by microbial fermentation of cellulose and other carbohydrates. Foregut VFA concentrations were in the upper range of values measured in a variety of other mammalian herbivores and were greater than those in foregut fermenting herbivores, such as cattle, sheep, kangaroos and sloths (Stevens and Hume, 2004). Further studies should investigate the contributions of foregut fermentation to the energy budget of rodents. Additionally, future studies should investigate the nutritional substrates in the foregut, as well as whether primarily bacteria, or commensal protozoa and fungi accomplish fermentation.

Concentrations of NH_3-N were also higher in the foregut than in the caecum. Ammonia is another index of fermentative digestion and is produced by the microbial degradation of proteins and amino acids. Furthermore, it is indicative of considerable recycling of endogenous urea. Many herbivores recycle urea to conserve nitrogen when feeding on low-nitrogen plant material (Stevens and Hume, 2004). The extent of urea recycling in the rodent foregut demands further study.

The concentration of microbial metabolites in the foregut is striking given the short residence time of food in this chamber. Many other foregut-fermenting mammals retain food in microbial chambers for extended periods of time to increase the digestion of fibre and liberation of nutrients for absorption in the small intestine (Stevens and Hume, 2004). In contrast, dietary items did not reside in the woodrat foregut for more than 1.5 h, yet we documented indicators of high microbial activity. The rates of microbial processes in the foregut may be rapid and demand further investigation. However, the microbiota may have been fermenting the simple sugars that are present in high abundance in cactus (El Kossori *et al.*, 1998), and not necessarily fibre. Future studies should investigate the substrates of the microbiota.

The dominant bacterial taxa in the woodrat gut varied across regions. The phyla Bacteroidetes and Firmicutes comprised the majority of communities across the gut, similar to other mammalian systems (Ley *et al.*, 2008). However, the abundance of Firmicutes was much

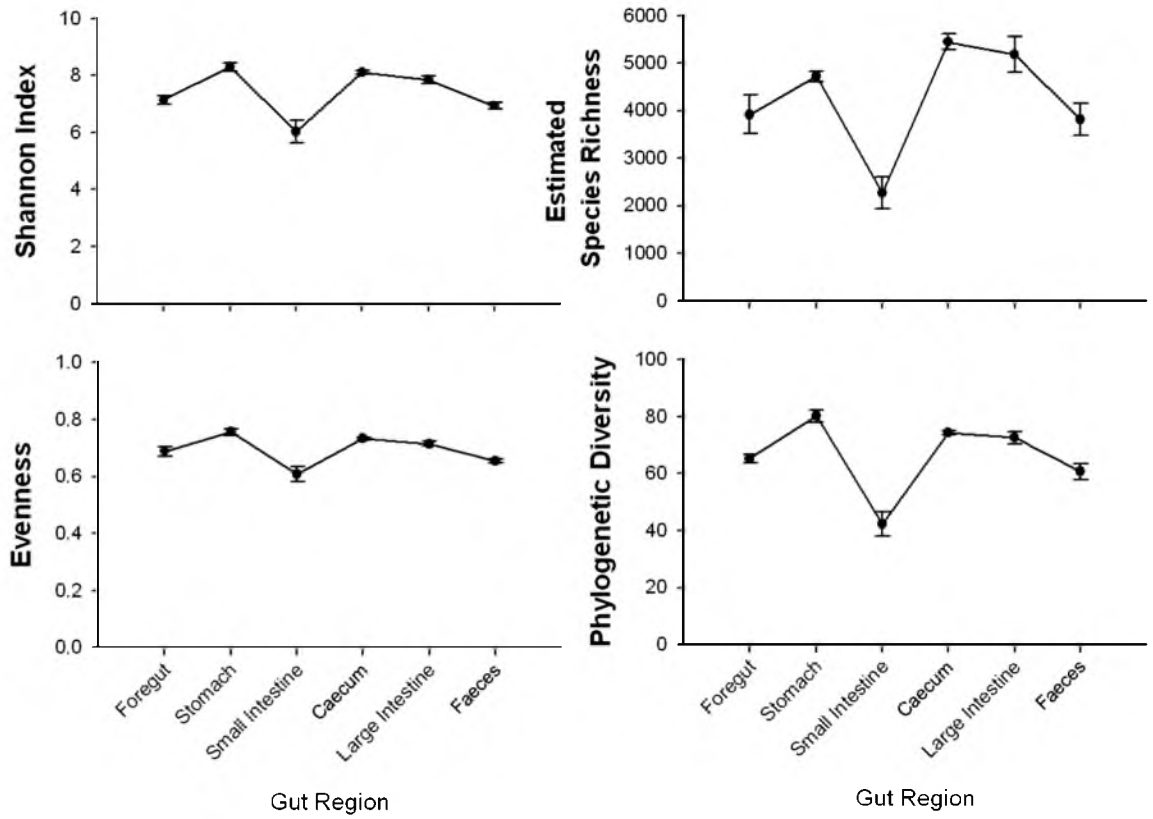

Fig. 4. Various diversity measurements of gut regions of *N. albigula*. The Shannon index is a metric that incorporates both richness and evenness. Estimated species richness was calculated using the Chao1 metric, which estimates the number of OTUs as the asymptote of a species accumulation curve. Evenness measures the variation in relative abundances of OTUs. A community where all OTUs are present in equal relative abundances has an evenness of 1. Phylogenetic diversity measures the cumulative branch lengths from randomly sampling OTUs from each sample.

reduced in the small intestine and faeces compared with other gut regions. At the genus level, *Oscillospira* was the most dominant identified genus of the woodrat gut. Although the functional capabilities of the uncultivable genus *Oscillospira* have not been determined, it is likely that it plays a role in fibre fermentation due to its presence in numerous rumen systems and its greater abundance in hosts that are fed fresh forage (Mackie *et al.*, 2003). This genus along with another cellulolytic genus, *Ruminococcus*, was present throughout the gut, but had low abundance in the small intestine. *Lactobacillus* comprised a substantial portion of the foregut community (~12.5%), but was not as abundant in other regions (< 4%). The dominance of *Lactobacillus* in the woodrat foregut has been documented for two other woodrat species, *Neotoma bryanti* and *Neotoma lepida* (Kohl and Dearing, 2012), and thus may be a common pattern in the gut communities of *Neotoma*. The genus *Lactobacillus* does not perform extensive fibre fermentation, but may be fermenting the simple sugars present in the foregut.

An immense amount of diversity existed in the woodrat gut in the form of rare and unidentified taxa. Approximately 50 identified genera collectively comprised less than 8% of the community of any gut region. We were unable to identify roughly 75–90% of sequences in any gut region at the genus level, and these sequences contained thousands of OTUs. Thus, the woodrat gut represents an extensive source of novel bacterial genera and species. This finding supports previous studies showing a high amount of novel sequences from the faecal microbial community of *N. bryanti* (Kohl *et al.*, 2011). Across mammals, herbivores exhibit the highest microbial diversity, and different mammalian clades harbour distinct communities (Ley *et al.*, 2008; Pope *et al.*, 2010). Other herbivorous rodents have not been

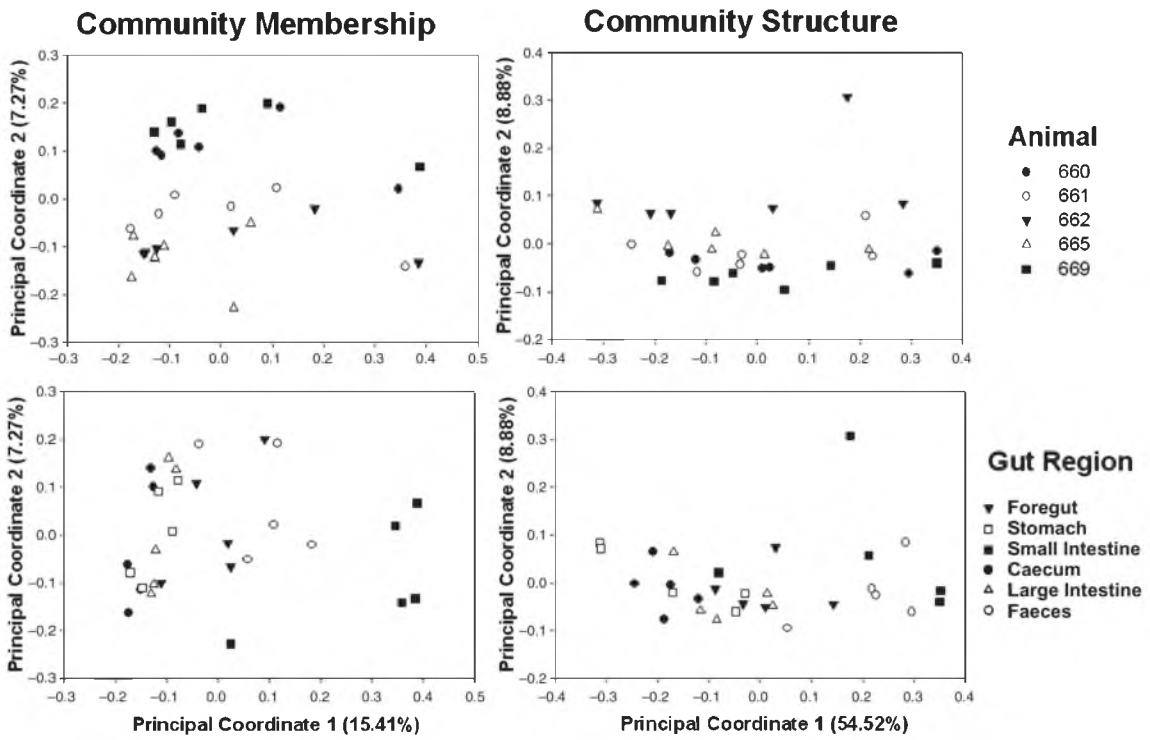

Fig. 5. Principal coordinate analysis of samples from *N. albigula*, grouped by either individual animal or woodrat gut region. Community membership, or the presence and absence of bacterial lineages, was primarily driven by gut region (adonis: $R^2 = 0.28$; $P = < 0.001$) and also by the individual animal ($R^2 = 0.05$; $P = 0.02$). Community structure, which takes relative abundances of taxa into account, was only driven by gut region ($R^2 = 0.55$; $P = < 0.001$) and not by individual animal ($R^2 = 0.04$; $P = 0.27$).

extensively studied in terms of microbial communities, which may explain this large amount of novelty.

Measurements of diversity varied significantly across gut regions. The small intestine harboured the lowest diversity in all metrics. This trend is likely due to the high activity of the immune system and the high flow rate within the small intestine (Lin, 2004). Surprisingly, the stomach often had the highest estimates of diversity despite its low pH and low density of live cells. However, 16S inventories do not differentiate live cells from dead cells, and so the stomach inventories may combine the resident live population of stomach microbes, as well as sequences from dead foregut microbes found within the stomach. The use of cell sorting based on cell wall integrity and 16S inven-

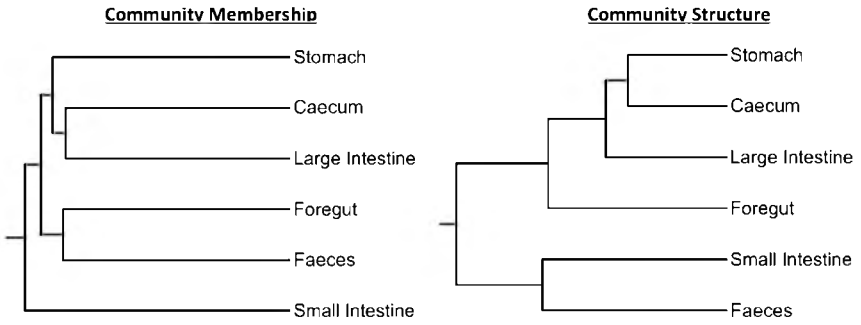

Fig. 6. UPGMA clustering of gut regions from *N. albigula* according to community composition and community structure. All nodes have jackknife support of 1.

tories of resulting 'live' and 'dead' populations would help understand the role of 'dead' cells in biodiversity estimates from whole samples.

Gut chamber influenced both bacterial community membership and structure. Regulation of bacterial communities among gut regions is likely driven by a number of factors, such as nutrient concentrations, immune responses, oxygen concentrations and flow rate. Additionally, the individual animal source determined bacterial community membership, suggesting that individuals have their own unique bacterial communities throughout gut regions. This observation may be driven by genotypic differences between individuals, such as immunity genes (Toivanen *et al.*, 2001) or unique production of glycans, yielding novel energy sources or binding areas (Hooper *et al.*, 2001). Alternatively, individual differences may be a result of founder effects. Mammals obtain their microbial community through contact with maternal faecal and vaginal microbes during the birthing process (Palmer *et al.*, 2007). The importance of this one-time exposure is highlighted by differences in the microbial community structure of conventionally and caesarean-delivered humans from infancy through childhood (Salminen *et al.*, 2004; Dominguez-Bello *et al.*, 2010). These founder effects might determine microbial communities and yield individual variation.

Bacterial communities were rarely similar between adjacent gut regions. Rather, communities were more similar between disparate gut regions. For example, the stomach and caecum have similar bacterial community structure, despite being separated by the small intestine and having radically different pH values. This suggests that there are complex interactions between various environmental characteristics (pH, flow rate, nutrient composition, immunity gene expression, etc.) that determine the community of any gut region.

Interestingly, the community in the faeces was not most similar to that of the large intestine in terms of community composition or structure. This difference could be due to exposure to oxygen upon leaving the gastrointestinal tract. Rather, faecal inventories were most similar to foregut communities in terms of bacterial community membership. We hypothesize that this similarity is driven by woodrats engaging in coprophagy (Kenagy and Hoyt, 1980). This behaviour may alter microbial community structure throughout the gut and may offer a constant source of microbial inoculation.

The results suggest that inventories from faecal material should be interpreted with caution. Researchers should refrain from extrapolating faecal inventories as indicators of microbial diversity of specific gut regions and should instead rely on direct sampling. In woodrats, there is a slight bias in terms of community structure. However, faecal inventories may still be useful to researchers. In woodrats, they seem to be somewhat indicative of the rest of the gut in terms of microbial community membership. Additionally, faecal inventories are informative when comparing treatment groups or species within a study, and provide the opportunity to conduct repeated sampling of an individual or collect non-lethal samples.

These data represent some of the first supporting the hypothesis that the rodent foregut serves to house an active microbial community, an idea that has existed for over a century (Toepfer, 1891; Carleton, 1973). The activity of this microbial population is striking given the small size and short residence time of this chamber. We hypothesize that the rodent foregut microbial community is important for several functions, such as the initial digestion of fibre, recycling of endogenous nitrogen and detoxification of dietary toxins.

Experimental procedures

Animals

Five individuals of *Neotoma albigula* were collected from Castle Valley, UT, on 17 October 2012. Animals were captured using Sherman live traps baited with peanut butter and oats, and were immediately transported back to the University of Utah Animal Facility. Woodrats were given cactus (*Opuntia spp.*) and juniper foliage (*Juniperus osteosperma*) *ad libitum* for one night. These are the dominant plants in Castle Valley and represent the natural diet (Macêdo and Mares, 1988). The following morning, woodrats were euthanized under CO_2 and dissected. We measured the mass of contents found within the foregut, stomach, small intestine, caecum and large intestine. Contents from each of these sections, as well as faecal samples, were collected and divided for various uses described below.

For measurements of gut transit time (described below), we used six individuals of *N. lepida* that were already in our colony, collected in July 2011 from Beaver Dam, Washington County, UT. Using these animals was preferable to collecting additional animals from their natural habitat. *Neotoma lepida* are similar to *N. albigula* in terms of size and gastrointestinal anatomy (Carleton, 1973). Also, congeneric rodents tend to display similar gut transit times (Kostelecka-Myrcha and Myrcha, 1964). The University of Utah Institutional Animal Care and Use Committee approved all experimental techniques under protocol 12–12010.

pH

Fresh contents from *N. albigula* were used immediately following dissection to measure the pH of various gut regions with an Omega Soil pH electrode (PHH-200).

Flow cytometry

Microbial density, as well as the proportion of live, injured and dead cells, were determined with the LIVE/DEAD BacLight Bacterial Viability and Counting Kit (Life Technologies, Grand

Island, NY, USA). Optimal filter settings were determined using a variation of the standard protocol. Woodrat faeces were cultured overnight in heart-brain infusion broth. Microbial cells were pelleted, and a subset was killed with 30 min incubation in isopropyl alcohol. Mixtures of live and dead cells were created to determine boundaries of gates for 'live' and 'dead' cells. During analysis of actual gut contents, cells falling between these regions were assigned as 'injured'.

Immediately following dissection, fresh gut contents from *N. albigula* were diluted with nine parts physiological saline and passed through 50 µm mesh cell strainers into tubes. Ten microlitre of filtrate was further diluted in 990 µl saline and stained with 3 µl of a mixture of components A and B from the LIVE/DEAD *Bac*Light Bacterial Viability and Counting Kit. Mixtures were incubated in the dark at room temperature for 30 min to allow for complete staining. Flow cytometric measurements were performed on a BD Biosciences FACSCanto II Flow Cytometer (San Jose, CA, USA) with 488 nm excitation. SYTO 9 and propidium iodide were measured through 530/30 and 695/40 filters respectively.

Volumetric measurement was determined by calibrating instrument flow rate with Spherotech Accucount Particles (ACFP 70–5; Lake Forest, IL, USA) as per manufacturer's guidelines. Briefly, clearly distinguishable fluorescent beads of a known concentration were acquired using the same flow rate as test samples to determine the volumetric flow rate per minute of the instrumentation.

Microbial metabolites

A portion of contents of the foregut and caecum from *N. albigula* were preserved for the analysis of VFAs and ammonia nitrogen (NH_3-N) by placing contents in an equal volume of 1 M NaOH or 1 M HCl respectively. Samples were frozen and transported to the University of Illinois. VFAs were measured using gas chromatography (Erwin *et al.*, 1961), and ammonia was determined by the indophenol method (Chaney and Marbach, 1962).

Gut transit time

To measure movement of digesta through various gut regions, we used six individuals of *N. lepida* fed with powdered rabbit chow (Harlan Teklad 2031, Madison, WI, USA) containing 2% (w/w) of coloured, inert plastic markers (1 mm diameter). Animals were given one colour of diet for a 24 h period, and colours were then switched every 1.5 h for 12 h during the animals' dark cycle. This interval was chosen because woodrats generally consume a meal every 1.5 h (Torregrosa *et al.*, 2012). Animals were then euthanized and dissected. The location of all plastic markers was determined, and we calculated the median, quartile, maximum and minimum locations through the gut for the contents of each meal. These values were averaged across individuals.

Bacterial inventories

Bacterial inventories were conducted on the samples collected from *N. albigula*. Frozen gut contents were thawed and whole DNA was isolated using a QIAamp DNA Stool Mini Kit (Qiagen, Germantown, MD, USA). Extracted DNA was sent to Argonne National Laboratories for sequencing. Bacterial inventories were conducted by amplifying the V4 region of the 16S rRNA gene using primers 515F and 806R, and paired-end sequencing on an Illumina MiSeq platform (Caporaso *et al.*, 2012).

Sequences were analysed using the QIIME software package (Caporaso *et al.*, 2010). Sequences underwent standard quality control and were split into libraries using default parameters in QIIME. Sequences were grouped into OTUs using UCLUST (Edgar, 2010) with a minimum sequence identity of 97%. The most abundant sequences within each OTU were designated as a 'representative sequence', and then aligned against the core set of Greengenes 13_5 (DeSantis *et al.*, 2006) using PyNAST (Caporaso *et al.*, 2009) with default parameters set by QIIME. A PH Lane mask supplied by QIIME was used to remove hypervariable regions from aligned sequences. FastTree (Price *et al.*, 2009) was used to create a phylogenetic tree of representative sequences. Sequences were classified using the Ribosomal Database Project (RDP) classifier with a standard minimum support threshold of 80% (Wang *et al.*, 2007). A comparison of taxon assignment by RDP and the Greengenes database showed similar results, with RDP performing slightly better. Sequences identified as chloroplasts or mitochondria were removed from analysis.

Several diversity measurements were calculated for each sample. We calculated the Shannon diversity index, a biodiversity measure that incorporates both richness and evenness. We also calculated an estimate of species richness (Chao1) and evenness, or how similar in abundance the OTUs in a sample are. However, these diversity metrics equally weight all OTUs regardless of phylogenetic relationships. Therefore, we calculated a measurement of phylogenetic diversity (Faith, 1992), which measures the cumulative branch lengths from randomly sampling OTUs from each sample. For each sample, we calculated the mean of 20 iterations for a subsampling of 7700 sequences.

We also compared community membership (presence or absence of bacterial lineages) and community structure (taking into account relative abundance of OTUs) of various gut regions. We calculated unweighted (for community membership) and weighted (for structure) UniFrac scores, and conducted PCoA (Hamady *et al.*, 2010). To compare the similarity of gut communities of different regions, we combined sequences within gut regions (across individuals) and conducted UPGMA hierarchical clustering of both unweighted and weighted UniFrac scores. Jackknife support of nodes in UPGMA trees were determined using default settings within QIIME. All sequences were deposited in NCBI's Sequence Read Archive under accession SRP022360.

Statistics

Microbial metabolites were compared between the foregut and caecum using paired *t* tests. Our sample size was insufficient to conduct repeated-measures analysis of variance (ANOVA) across all six gut regions. Relative abundances of bacterial taxa were compared across gut regions using ANOVA, and *P* values underwent Bonferroni correction. Biodiversity metrics were compared across gut regions within

an individual using the Friedman test, which is a non-parametric test for one-way repeated measures analysis and allows for a more conservative estimate of differences between gut regions. We investigated the effects of individual animal and gut region on bacterial community membership and structure using the adonis function in QIIME with 999 permutations.

Acknowledgements

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Conflict of interest: The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Percentage of live, injured and dead microbial cells in each gut region of *N. albigula*.

CHAPTER 7

EXPERIENCE MATTERS: PRIOR EXPOSURE TO PLANT TOXINS ENHANCES DIVERSITY OF GUT MICROBES IN HERBIVORES

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LETTER

Experience matters: prior exposure to plant toxins enhances diversity of gut microbes in herbivores

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Abstract

For decades, ecologists have hypothesised that exposure to plant secondary compounds (PSCs) modifies herbivore-associated microbial community composition. This notion has not been critically evaluated in wild mammalian herbivores on evolutionary timescales. We investigated responses of the microbial communities of two woodrat species (*Neotoma bryanti* and *N. lepida*). For each species, we compared experienced populations that independently converged to feed on the same toxic plant (creosote bush, *Larrea tridentata*) to naive populations with no exposure to creosote toxins. The addition of dietary PSCs significantly altered gut microbial community structure, and the response was dependent on previous experience. Microbial diversity and relative abundances of several dominant phyla increased in experienced woodrats in response to PSCs; however, opposite effects were observed in naïve woodrats. These differential responses were convergent in experienced populations of both species. We hypothesise that adaptation of the foregut microbiota to creosote PSCs in experienced woodrats drives this differential response.

Keywords

Community structure, gut microbiota, mammalian herbivores, *Neotoma*, symbiotic microbes.

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INTRODUCTION

Mammalian herbivores are repeatedly faced with the challenge of consuming food sources that are low in nitrogen and high in indigestible material, such as fibre. To overcome these challenges, herbivores maintain communities of symbiotic microbes that increase the nutritive quality of foliage through the synthesis of essential amino acids or fermentation of fibre (Muegge *et al.* 2011). Herbivores maintain microbial communities that differ drastically from other mammals in terms of both composition and function, including an increased abundance of species with genes associated with the above processes (Ley *et al.* 2008; Muegge *et al.* 2011). However, plants also produce a wide array of plant secondary compounds (PSCs) that discourage or reduce consumption by altering the homeostasis of the herbivore (Dearing *et al.* 2005). In addition, PSCs, and not nutrient constraints, may be more important in limiting the ability of mammalian herbivores to specialise on certain plant species (Dearing *et al.* 2000).

Nearly four decades ago, Freeland and Janzen predicted that a mammalian herbivore's exposure to PSCs sculpted its gut microbial community (Freeland & Janzen 1974). The intense selective pressure of these potentially lethal and antibacterial compounds on herbivore hosts and their microbiota should favour a certain composition of microbes associated with detoxification and tolerance (Barboza *et al.* 2010). Work on herbivorous insects lends support to this hypothesis (Kelley & Dobler 2011). However, to date, only two studies have addressed this issue in mammalian herbivores (Jones & Megarrity 1986; Sundset *et al.* 2010). These studies focused on particular species of microbes or individual PSCs, and not alterations in total community structure with respect to complex mixtures of PSCs. The hypothesis that herbivore experience with PSCs influences mammalian gut microbial communities has not yet been critically evaluated using both experimental and comparative approaches.

To address this gap in our understanding of the effects of PSCs on gut microflora, we investigated the gut microbial communities from populations of two species of woodrats, Bryant's woodrat (*Neotoma bryanti*) and the desert woodrat (*N. lepida*). Both of these species have independently converged on the same PSC rich diet of creosote bush, (*Larrea tridentata*). The dietary strategies and evolutionary histories of woodrats have been well documented, particularly for *N. bryanti* and *N. lepida* (Atsatt & Ingram 1983; Dial 1988; Patton *et al.* 2007). These two species diverged roughly 1.6 million years ago; their diets at the time of speciation are unknown (Patton *et al.* 2007). However, both species underwent a radical dietary shift 17 000 years ago when creosote naturally invaded their habitat (Hunter *et al.* 2001). Juniper (*Juniperus spp.*) had been the predominant shrub in the area for at least the past 40 000 years, but was extirpated by natural changes in climate (Van Devender & Spaulding 1979; Hunter *et al.* 2001). The leaves of creosote bush are covered in a phenolic-rich resin that can comprise 10–25% of the dry mass (Mabry *et al.* 1977). Creosote resin is a complex mixture of hundreds of chemical products, including phenolics, O-methylated flavones and flavonols, catechols, vinyl ketones and saponins (Mabry *et al.* 1977). The majority of this resin is composed of nordihydroguaiaretic acid, a phenolic compound that causes kidney cysts in lab rats (Goodman *et al.* 1970). Due to creosote's limited range, there are populations of woodrats that have never experienced creosote bush ('naïve populations'), as well as those that have up to 17 000 years of ecological experience with creosote ('experienced populations'). Experienced populations consume roughly 75% creosote bush in the wild (Karasov 1989), and are able to consume 25% more creosote resin in the laboratory compared with naïve populations (Mangione *et al.* 2000). Currently, naïve populations of *N. bryanti* feed primarily on cactus (*Opuntia occidentalis*) and sage (*Salvia spp.*), which produce low molecular weight PSCs such as oxalates or diterpenes, and thus have different plant chemistry profiles compared with creosote bush (Atsatt & Ingram 1983; Stintzing

& Carle 2005; Abreu *et al.* 2008). Naïve *N. lepida* persist in the ancestral habitat of juniper woodlands, which contain monoterpenes, phenolics and condensed tannins (Adams *et al.* 1981; Utsumi *et al.* 2009). Thus, populations of both species feed on diets with unique PSC profiles.

In addition, the gut morphology of woodrats makes them ideal herbivores in which to investigate interactions between PSCs and microbial communities. To deal with the high fibre content of plant material, woodrats, like many rodents, maintain a hindgut fermentation chamber, known as the caecum. However, woodrats also have highly segmented stomach morphology, the function of which is unknown (Carleton 1973). Woodrats possess a foregut chamber (termed 'pregastric stomach' in Kohl *et al.* 2011) that consists of non-secretory epithelium (Carleton 1973), and so should maintain a more neutral pH and facilitate more microbial growth compared with the acidic, gastric chamber. Interestingly, the microbes present in the faeces of woodrats more closely resemble the patterns observed in distantly related, foregut fermenting Artiodactyls rather than closely related rodents, suggesting that this foregut structure and its resident microbes could be important to host physiology (Kohl *et al.* 2011). Microbial detoxification would be most beneficial to a host if it were to occur proximally in the gastrointestinal tract to permit detoxification prior to absorption in the small intestine (Freeland & Janzen 1974). Thus, the foregut of woodrats could be critical to facilitating growth of microbes important for detoxification of PSCs.

We tested the hypothesis that the foregut of woodrats houses a microbial community. In addition, we investigated the determinants of microbial community structure when all individuals were placed on a novel, non toxic diet. Finally, we examined whether evolutionary history or convergence on similar natural diets of creosote bush affected the response of the microbial community to the PSCs. Together, these studies represent the first comparative and experimental investigation into the interactions between PSCs and gut microbes in wild herbivores.

MATERIAL AND METHODS

Animal collection and maintenance

Details of trapping locations and dates for each population are described in Appendix S1 in Supporting Information. All animals used in our experiment were collected with Sherman live traps at locations given in Appendix S1. Woodrats were transported to the University of Utah Department of Biology Animal Facility and housed in individual cages (48 × 27 × 20 cm) under a 12:12-h light:dark cycle, with 28 °C ambient temperature and 20% humidity.

Diet treatments

Prior to experimentation, animals were maintained on a diet of high-fibre rabbit chow (Harland Teklad formula 2031). During experimentation, animals were fed the same chow except in a powdered form to prevent caching of food. Four individuals from each population served as control animals and were fed powdered rabbit chow in cages for 8 days. Four individuals from each population were fed the control diet for 3 days, followed by the same diet with increasing amounts of creosote resin (1 and 2% creosote resin for 2 and 3 days respectively). A diet of 2% creosote resin is the maximum concentration at which naïve individuals maintain body mass (Mangione *et al.* 2000). Moreover, it represents a tolerable diet for experienced individuals, as they consume a diet containing ~ 7.5% resin in the wild (Mabry *et al.* 1977; Karasov 1989). The 5-day experimental treatment is sufficient for observing changes in the microbial community given the retention time for woodrats (Karasov *et al.* 1986).

To prepare diet treatments containing creosote resin, creosote leaves were collected from trapping sites and frozen at −20 °C prior to resin extraction. We performed surface extractions from creosote leaves using techniques adapted from Mabry *et al.* (1977). Resin was extracted by soaking leaves in acetone (1:6, wet leaf mass:volume solvent) for 45 min. Solvent and resin were filtered (Whatman filter paper grade 1) to remove large particles and evaporated using a rotovap until the resin was highly viscous, at which point it was transferred to a vacuum pump for 48 h to remove any remaining acetone. Extracted resin was stored at −20 °C prior to use.

Creosote diet treatments were prepared by dissolving the appropriate amount of resin in a volume of acetone equal to 25% of the dry weight of ground rabbit chow to which it was added. Control diet (0%) was prepared by adding an identical ratio of acetone, without creosote resin. Acetone was evaporated from all diets in a fume hood and complete evaporation was confirmed gravimetrically. Acetone is a common disinfectant and sterilizing agent (Drews 1977).

Following diet treatments, animals were euthanised under CO_2 and immediately dissected. Contents of the foregut were removed and frozen at −80 °C until DNA isolation.

DNA isolation and sequencing

Foregut contents were thawed on ice and a small amount (~ 25 mg) was incubated with 180 µL enzymatic lysis buffer at 37 °C for 30 min to degrade the cell walls of gram positive bacteria. The lysis buffer consisted of 20 mM Tris–Cl, pH 8.0, 2 mM sodium EDTA and 1.2% TritonX-100 dissolved in deionized water, with 20 mg mL^{-1} lysozyme added before use. DNA was extracted from faecal material using a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA). Microbial 16S rRNA genes were amplified and sequenced using tag-encoded FLX amplicon pyrosequencing (TEFAP) using the universal primers 28F (5′-GAGTTTGATCNTGGCTCAG-3′) and 519R (5′ACCGCGGCTGCTGGCAC 3′). TEFAP was conducted by Research and Testing Laboratories (Lubbock, TX, USA), with methods described in detail in Sun *et al.* (2011).

Sequence analysis

Sequences were analysed using the QIIME software package (Caporaso *et al.* 2010). Sequences underwent standard quality control, and were removed from analysis if they lacked the primer sequence or had either quality scores < 25, read lengths < 200 bp or ambiguous base reads. Remaining sequences were assigned to specific samples using 8-bp barcodes assigned by Research and Testing Laboratories. Sequences were grouped into operational taxonomic units (OTUs) using UCLUST (Edgar 2010) with a minimum sequence identity of 99%. The use of 99% minimum sequence identity over 97% allows for better phylogenetic resolution. The most abundant sequences within each OTU were designated as a 'representative sequence', and then aligned against the Greengenes core set (DeSantis *et al.* 2006) using PyNAST (Caporaso *et al.* 2009) with default parameters set by QIIME. A PH

Lane mask supplied by QIIME was used to remove hypervariable regions from aligned sequences. FastTree (Price *et al.* 2009) was used to create a phylogenetic tree of representative sequences.

Diversity of the woodrat foregut, and determinants of diversity on a non-toxic diet

To investigate diversity within the woodrat foregut, OTUs were classified using the Ribosomal Database Project classifier with a the standard minimum support threshold of 80% (Wang *et al.* 2007). Sequences identified as chloroplasts were removed from analysis. Relative abundances of taxa were averaged across all individuals to give a representation of taxa residing in the woodrat foregut. In addition, we compared relative abundances of the dominant phyla from individuals fed the control diet using a two way ANOVA with species and experience (naïve vs. experienced) as the main effects. Insignificant interactions were removed from the final analysis.

Effect of creosote resin on microbial community representation

We compared community memberships (presence or absence of lineages, and not their relative abundances) of treatment groups. We calculated unweighted UniFrac scores and conducted Principal Coordinates Analysis. To investigate how similar treatment groups were to one another, we combined individuals within treatments after normalising for the number of sequences per sample. Diversity shared between treatment groups (β diversity) was measured using the UniFrac metric, which utilises the fraction of branch length shared between two samples in the phylogenetic tree created from all representative sequences. The UPGMA (Unweighted Pair Group Method with Arithmetic Mean) hierarchical clustering of treatment groups was carried out using unweighted UniFrac distances.

Effect of creosote resin on microbial community structure

Several α diversity measurements were calculated for each sample. We calculated the Shannon Diversity Index, a biodiversity measure that incorporates both richness and evenness. We calculated evenness, or how similar in abundance the OTUs in a sample are, as well as Chao1, which estimates the asymptote on a species accumulation curve to estimate OTU richness. However, these diversity metrics weight all OTUs equally regardless of phylogenetic relationships. Therefore, we calculated a measurement of phylogenetic diversity (Faith 1992), which measures the cumulative branch lengths from randomly sampling OTUs from each sample. For each sample, we calculated the mean of 20 iterations for a sub-sampling of 1441 sequences and calculated the mean for each treatment.

To evaluate the role of previous experience with creosote on microbiota diversity, relative abundances of taxa, Shannon Index, evenness, Chao1 and Phylogenetic Diversity scores were compared using three-factor ANOVAs, using species, ecological experience and diet as main effects. If interaction terms were insignificant they were removed from analysis. Statistical analyses were conducted in JMP 9.0.

Data deposition

The bacterial 16S rRNA gene sequences from our effort were deposited in the GenBank Sequence Read Archive under accession number SRA048649.1.

RESULTS

Diversity of the woodrat foregut

We obtained a total of 144 863 high quality microbial 16S rRNA sequences (4527 ± 334 sequences per individual) across all samples from the woodrat foregut. These high quality reads were obtained after quality control removed roughly 0.4% of original sequence reads, and alignment and filtering for chloroplast removed ~ 20% (Appendix S2). Clustering of sequences with 99% sequence similarity identified 4825 OTUs. Within these OTUs, we identified seven microbial phyla (Table 1) The dominant genera present were *Lactobacillus* (73.1 ± 4.3%) and *Allobaculum* (2.5 ± 0.1%).

Determinants of diversity on a non-toxic diet

The two host species had foregut microbial communities with distinct characteristics when feeding on the non-toxic laboratory diet (Fig. 1). *N. lepida* hosted significantly fewer Firmicutes and more Bacteroidetes and Actinobacteria compared to *N. bryanti* (species effect: $P < 0.01$ for all phyla; Appendix S3).

Responses of the foregut microbiota to creosote resin

Community membership (the presence of lineages) of the foregut was determined by experience with creosote resin, as well as host species association. Populations of *N. lepida* share similar microbial community memberships (Fig. 2; Appendix S4). Interestingly, the microbiota of experienced *N. bryanti* was more similar to that of *N. lepida* (regardless of experience) than naïve *N. bryanti* (Fig. 2; Appendix S4). Diet treatment in the laboratory did not affect community membership, i.e., similar gut microbes were present in woodrats regardless of feeding on creosote resin (Fig. 2; Appendix S4).

Changes in community structure (relative abundances of lineages) due to addition of PSCs depended on previous ecological experience with creosote bush. We found significant diet by experience interactions for the relative abundances of the two dominant phyla residing in the woodrat foregut (Figs 1 and 3, Appendix S5). Experienced individuals had a lower relative abundance of Firmicutes and a greater relative abundance of Bacteroidetes when fed creosote resin, whereas naïve individuals showed an inverse pattern (Figs 1 and 3; Appendix S5). There was a trend for increased relative abundance of Actinobacteria when woodrats fed on creosote, particularly in experienced individuals (Appendix S5).

Similar significant interactions between diet treatment and ecological experience were observed across several of the biodiversity

Table 1 Dominant phyla residing in the woodrat foregut

Phylum	Relative abundance
Firmicutes	79.8 ± 3.7
Bacteroidetes	11.5 ± 2.2
Actinobacteria	6.5 ± 1.6
TM7	0.3 ± 0.1
Proteobacteria	0.3 ± 0.1
Fusobacteria	0.01 ± 0.006
Spirochaetes	0.01 ± 0.006
Other	1.57 ± 1.08

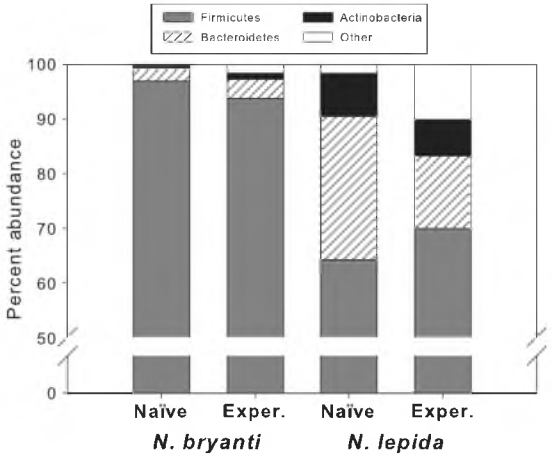

Figure 1 Relative abundances of microbial taxa from foreguts of four woodrat populations fed the control diet.

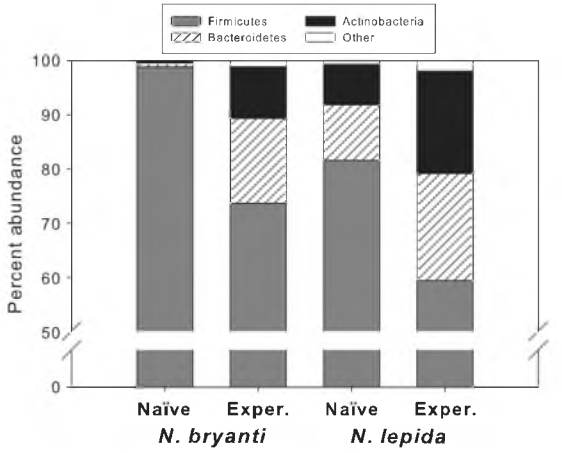

Figure 3 Relative abundances of microbial taxa from woodrat foreguts of four woodrat populations fed the creosote diet.

measurements, whereas the main effects of diet and ecological experience alone had no influence on any estimates of biodiversity (Table 2; Appendix S6). Microbial diversity was 20% greater in experienced individuals fed creosote resin compared with the control diet as measured by the Shannon Index, whereas naïve individuals exhibited ~ 5% lower diversity (Table 2; Fig. 4A). This effect is most likely driven by evenness of microbial species, which showed similar patterns (Table 2; Fig. 4B), whereas estimates of species richness (Chao1) did not differ (Table 2; Fig. 4C). Phylogenetic diversity also showed a significant diet by experience interaction (Table 2), such that phylogenetic diversity was higher in experienced individuals fed creosote resin compared with the control diet, whereas naïve individuals exhibited lower phylogenetic diversity (Fig. 4D). Thus, the overall response of individuals to dietary creosote resin seems to be largely influenced by previous ecological experience.

DISCUSSION

Plant secondary compounds represent a persistent challenge to mammalian herbivores, yet their interactions with the gut microbiota have been largely understudied in comparison with other dietary components, such as high fibre or low nitrogen. In this study, we inventoried the microbial community of two mammalian herbivores and investigated how evolutionary history and ecological exposure to PSCs influences the microbial community membership, as well as changes in community structure in response to dietary PSCs. This work represents the first experimental study on how the microbial communities of wild herbivores respond to dietary toxins. Below, we discuss how PSCs seem to have shaped the microbial communities of the woodrat foregut and how differential responses might have functional consequences for herbivores attempting to utilise new food sources.

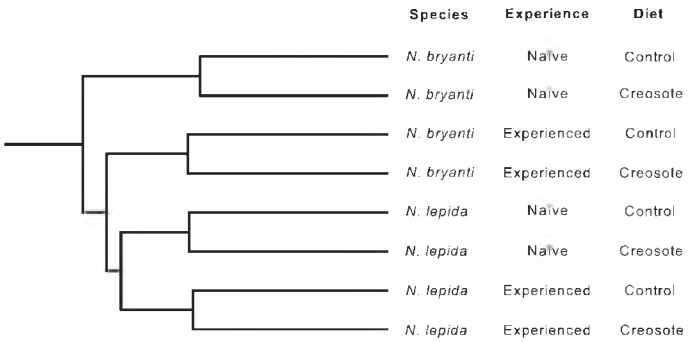

Figure 2 Results of UPGMA hierarchical clustering of treatment groups using unweighted UniFrac distances. Operational taxonomic unit abundances were normalised per individual and combined by treatment.

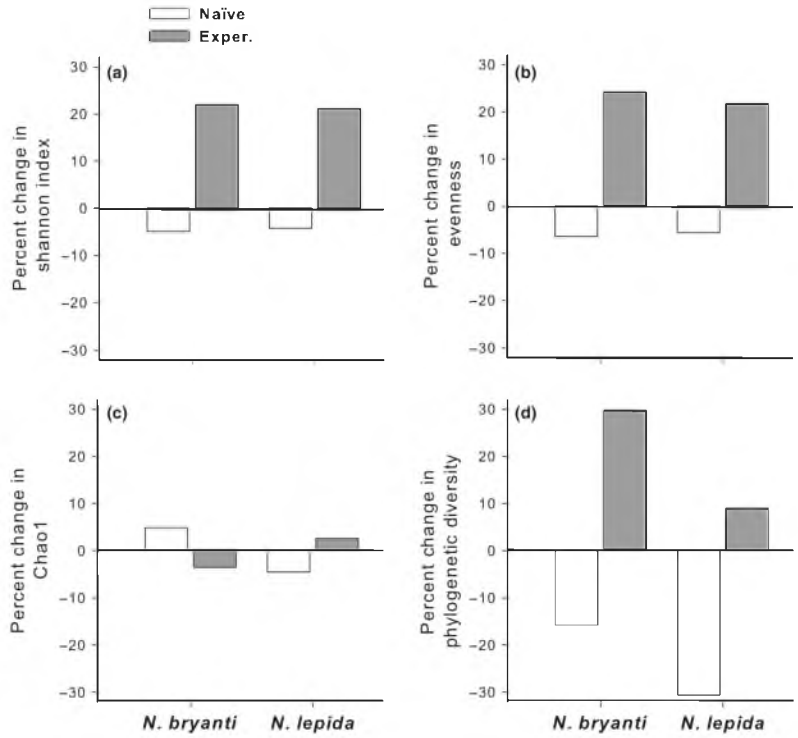

Figure 4 Per cent change in α diversity indices from control diet to feeding on a creosote diet. Data for each dietary treatment are presented in Table 2. Details of statistical analysis are presented in Appendix S6.

Table 2 Means ± 1 SEM and significant effects for measurements of α diversity indices from woodrats on control and creosote diets. Insignificant interactions were removed from analysis

	Control				Creosote				
	Neotoma bryanti		*Neotoma lepida*		*Neotoma bryanti*		*Neotoma lepida*		
	Naïve	Exper.	Naïve	Exper.	Naïve	Exper.	Naïve	Exper.	Effects†
Shannon index	5.53 ± 0.25	5.19 ± 0.60	6.40 ± 0.37	5.40 ± 0.52	5.26 ± 0.47	6.33 ± 0.35	6.13 ± 0.10	6.54 ± 0.03	S*; E × D*
Evenness	0.62 ± 0.03	0.58 ± 0.06	0.71 ± 0.05	0.60 ± 0.05	0.58 ± 0.05	0.72 ± 0.04	0.67 ± .01	0.73 ± 0.01	E × D**
Chao1	850.1 ± 93.3	803.9 ± 74.2	985.3 ± 125.1	864.2 ± 94.6	891.4 ± 62.5	775.5 ± 90.7	940.6 ± 2.9	886.7 ± 46.9	
Phylogenetic diversity	9.97 ± 1.37	11.51 ± 1.82	18.78 ± 1.42	13.95 ± 1.46	8.39 ± 1.37	14.93 ± 2.09	13.03 ± 1.17	15.20 ± 0.99	S**; S × E*; E × D**

†Indicates which treatment effects are significant (S = species, E = experience, D = diet). Interactions are denoted with '×'. Details of statistical analysis are presented in Appendix S6. Asterisks indicate the level of significance, determined by ANOVA.
*$P < 0.05$; **$P < 0.005$.

Diversity of the woodrat foregut

The microbial community of the woodrat foregut consists primarily of Firmicutes (~ 80%). This high representation of Firmicutes drastically differs from that of other foregut structures such as the bovine rumen, where Firmicutes contribute to ca. 30% of the microbial population (Callaway *et al.* 2010). As the functional repertoire of microbial genes can be predicted in part by microbial community structure (Muegge *et al.* 2011), and because of the differences between the microbiota of the woodrat foregut to those present in other foreguts, the function of the woodrat foregut is unlikely to be analogous to that of the bovine rumen. In addition, the smaller size of woodrat foregut coupled with probable shorter residence time of material in comparison to that of the caecum

(Kohl *et al.* 2011), suggests that the foregut is unlikely to play a key role in cellulolytic fermentation.

Rather, our results support the hypothesis that the woodrat foregut serves as a detoxification chamber. We found seven microbial phyla, many of which are known to play a role in toxin metabolism. The most predominant genus, *Lactobacillus*, degrades plant phenolics (Rodriguez *et al.* 2008) and dissociates tannin-protein complexes (Shimada *et al.* 2006). Moreover, this genus seems to be essential for the ability of the Japanese wood mouse (*Apodemus speciosus*) to feed on polyphenolic-rich acorns (Shimada *et al.* 2006). The phylum Actinobacteria is well known for its biotransformation abilities, such as oxidation of small cyclic hydrocarbons similar in structure to PSCs (Donova 2007). For example, Actinobacteria are thought to be important in the degradation of plant phenolics in the guts of termites (Le Roes-Hill *et al.* 2011). It is also possible that other less abundant microbes play a role in detoxification, or perhaps other functions related to detoxification processes, such as free-radical scavenging. In the future, we plan to conduct metagenomic sequencing to better understand the function of the woodrat foregut.

Determinants of diversity on a non-toxic diet

Our results show that species maintain unique assemblages of microbes, even when housed in the same animal room and after experiencing similar laboratory conditions. At this point, we cannot speculate how these communities change from natural to laboratory conditions. These distinct assemblages could be the products of unique host-microbial interactions among populations. For example, some hosts produce molecules (glycans) that enhance particular microbial species and generate unique communities (Hooper & Gordon 2001). Alternatively, as these animals were captured in the wild, we cannot exclude the possibility that the distinct assemblages are the legacy of unique founder microbial populations from each habitat.

Determinants of microbial community membership

Diet did not significantly alter microbial community membership, as shown by UPGMA hierarchical clustering. This suggests that the addition of creosote resin does not add or remove a significant number of microbial lineages from the woodrat foregut. Rather, experience with PSCs and evolutionary history determined foregut community membership. This is in contrast to herbivorous insects, where PSC class, and not evolutionary history, determine microbial diversity (Kelley & Dobler 2011). This difference may be due to the fact that mammals inherit their microbial communities from their mothers, whereas insects seem to acquire microbes from the environment (Kelley & Dobler 2011). Individuals of *N. lepida*, regardless of experience, shared similar microbial communities. However, experienced *N. bryanti* had microbial communities more similar to *N. lepida* (regardless of experience) rather than naïve *N. bryanti*. This pattern could also be the result of similar PSC profiles in the diets of the experienced woodrats and naïve *N. lepida*. Both juniper (the diet of naïve *N. lepida*) and creosote contain high concentrations of phenolics (Mabry *et al.* 1977; Utsumi *et al.* 2009). Thus, the microbial communities of *N. lepida* and experienced *N. bryanti* may be specialised for degrading phenolics. However, juniper also contains high concentrations of monoterpenes (Adams *et al.* 1981), and so it is puzzling why we find such similar communities between naïve and experienced *N. lepida*. However, experienced *N. lepida* retain the ability and detoxification machinery to feed on their ancestral diet of juniper (Magnanou *et al.* 2009), and may have also retained juniper-specific microbes while adding several microbial taxa to aid in the metabolism of creosote toxins.

In contrast, the diet of naïve *N. bryanti* contains low-molecular weight PSCs, such as oxalates or diterpenes (Stintzing & Carle 2005; Abreu *et al.* 2008). Therefore, the diet switch that occurred within *N. bryanti* represents a larger change in PSC profiles compared with *N. lepida*, and may have selected for the disparate communities observed. The microbial community of naïve *N. bryanti* may specialise in the degradation of the PSC classes it naturally encounters, such as oxalates. Indeed, microbes are important for oxalate degradation in another woodrat species, *N. albigula* (Shirley & Schmidt-Nielsen 1967). However, we cannot exclude the idea that the length time in captivity contributed to the unique microbial community observed in naïve *N. bryanti*.

Response to creosote resin

The response of the microbiota to creosote PSCs depended largely on previous experience with creosote. For example, experienced woodrats fed on creosote resin exhibit lower relative abundances of Firmicutes and higher relative abundances of Bacteroidetes compared with the control diet. In addition, the relative abundance of Actinobacteria was higher in experienced woodrats fed creosote, but showed less change in naïve woodrats. Likewise, biodiversity was higher in the foreguts of experienced woodrats fed creosote resin, but lower in naïve. As the functions of collective microbial genes can be predicted by microbial community structure (Muegge *et al.* 2011), we predict that this shift in microbial diversity is matched by a shift in microbiome function. Microbial biodiversity is often positively correlated with ecosystem function (Bell *et al.* 2009). It is possible that the ingestion of a novel toxin skews the relative abundance of microbial genes away from ideal representation, such as less representation of important fermentation or amino acid synthesis genes, which could then compromise the nutritional status of the animal. Thus, adaptation of the microbiota to specific PSC profiles may limit an herbivore's ability to shift diets quickly or utilise novel plant species. This specialisation within the microbiota, and impaired function due to novel PSCs may be a mechanism by which specialist herbivores are unable to utilise plants with novel PSCs as effectively as generalist herbivores (Sorensen *et al.* 2005).

The mechanism of these differential responses still remains unclear. They could be driven in part by host physiology, such that experienced hosts change the profile of their glycan or mucin diversity in response to creosote resin to select a certain microbial population. In addition, responses could have been driven by various mechanisms through which the microbiota of experienced woodrats have become adapted to an environment rich in PSCs, such as descent with modification within the microbiota, or more likely horizontal transfer of genes from transient microbes to the resident microbiota. Indeed, horizontal gene transfer within gut-associated bacteria occurs at a rate 25 × higher than other bacteria (Smillie *et al.* 2011), and is important for the acquisition of new metabolic capabilities to allow gut microbes to cope with plant compounds (Hehemann *et al.* 2010; Nelson *et al.* 2010). Through these mechanisms, representatives of Bacteroidetes and Actinobacteria residing

within experienced hosts may have accumulated genes important for the detoxification of creosote, allowing them to increase in relative abundance when the host is consuming creosote resin.

It is also noteworthy that the responses of experienced herbivores are convergent across species. Other studies have documented that nutritive factors cause convergence in the microbial communities of herbivores from disparate mammalian lineages (Ley *et al.* 2008; Muegge *et al.* 2011). Our study reveals that PSCs appear to have selective pressures similar to that of nutrients on the microbial communities of herbivores. Community convergence is exhibited by similar changes in biodiversity measures and relative abundances of dominant phyla in response to creosote PSCs in the experienced woodrat microbial communities. Metagenomic comparisons may elucidate whether the microbial communities of each woodrat species have convergently or uniquely adapted to PSCs at the microorganism or gene level.

Our results highlight the importance of considering ecological experience when investigating microbial responses to PSCs and perhaps other xenobiotics. Recently, there has been a large effort to use PSCs as modifiers of the rumen ecosystem in cattle and sheep due to the increasing restrictions on growth-promoting antibiotics in agriculture (Patra & Saxena 2009). In addition, the gut microbiota seems to play a large role in human obesity and modification of the gut microbiota through the use of PSCs is being investigated as a potential treatment (Rastmanesh 2011). However, our study shows differential responses within the same host species, and so such endeavours should consider host experience when conducting experiments or translating results to other systems.

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AUTHORSHIP

KDK conducted the experiment, participated in data interpretation and wrote the first draft of the manuscript. MDD participated in the data interpretation, contributed to revisions of the manuscript and oversaw the project.

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CHAPTER 8

THE GUT MICROBIOTA ALLOW INGESTION AND METABOLISM OF CHEMICALLY DEFENDED PLANTS

Abstract

The foraging ecology of mammalian herbivores is strongly shaped by plant secondary compounds (PSCs) that act to defend plants against herbivory. Conventional wisdom holds that gut microbes facilitate the ingestion of toxic plants; however, this notion lacks empirical evidence. I investigated the gut microbiota of the desert woodrat (*Neotoma lepida*), some populations of which specialize on highly toxic creosote bush (*Larrea tridentata)*. Here, I demonstrate that the gut microbiota is crucial in allowing herbivores to feed on toxic plants. When woodrats were fed creosote toxins, the population structure of the gut microbiome changed to facilitate an increase in abundance of genes that metabolize toxic aromatic compounds. Additionally, woodrats were unable to feed on creosote toxins after the microbiota was removed with a broad-spectrum antibiotic. Last, I demonstrate that tolerance to toxins can be conferred to naïve hosts through microbial transplants. Transplant recipients exhibited increased toxin tolerance and unique detoxification routes. These results demonstrate that microbes can enhance host tolerance to PSCs and therefore expand the dietary niche breadth of wild mammalian herbivores, and host tolerance to toxins can occur rapidly through the acquisition of novel

gut microbes. Therefore, microbial detoxification represents an accelerated mechanism by which herbivores may rapidly adapt to novel and more potent PSCs brought about by environmental changes. The potential for microbes to assist herbivores in consuming dietary toxins represents an uncharted frontier in our understanding of plant-herbivore interactions.

Introduction

Among mammals, herbivores comprise the most abundant feeding guild (Price et al., 2012), playing critical roles in shaping ecosystem structure (Martin and Maron, 2012), and serving as essential resources to humans as livestock. A major determinant of the dietary niche breadth of mammalian herbivores is tolerance to plant secondary compounds (PSCs) (Dearing et al., 2000; Moore and Foley, 2005). Nearly four decades ago, renowned ecologists Freeland and Janzen described a suite of strategies employed by mammalian herbivores to overcome challenges posed by PSCs (Freeland and Janzen, 1974). One of those strategies, the use of microbial detoxification, has been generally overlooked in research on plant-herbivore interactions (Dearing et al., 2005). The handful of documented examples of microbial detoxification in mammalian herbivores are restricted to agricultural animals feeding on a single toxic compound (Jones and Megarrity, 1986; Sundset et al., 2010). Conversely, wild mammalian herbivores forage on plants producing myriad chemical defenses (Moore and Foley, 2005).

I investigated the microbial communities of the desert woodrat (*Neotoma lepida*). Approximately 17,000 years ago, natural climate change facilitated the invasion of creosote bush into the southern portion of the range of the desert woodrat (Van Devender and Spaulding, 1979; Hunter et al., 2001). The leaves of creosote bush are covered in a

phenolic-rich resin composed of a complex mixture of hundreds of chemical products, including phenolics, *O*-methylated flavones and flavonols, catechols, vinyl ketones, and saponins, which, together comprise up to 25% of the dry mass of the plant (Mabry et al., 1977). The majority of creosote resin is comprised of nordihydroguaiaretic acid (NDGA), a phenolic compound that causes kidney cysts and liver damage in laboratory rodents (Goodman et al., 1970; Lambert et al., 2002). In the Mojave desert of the USA, populations of *N. lepida* feed primarily on creosote bush (Karasov, 1989), and each day consume doses of resin that would be lethal to laboratory mice (Rios et al., 2008). However, creosote bush did not invade the adjacent Great Basin desert, resulting in naïve populations of *N. lepida* that lack ecological and evolutionary experience with creosote and feed instead on the ancestral diet of juniper. In the laboratory, woodrats from experienced (Mojave) populations can consume roughly 25% more creosote resin than those from naïve (Great Basin) populations (Mangione et al., 2000). The experienced population has higher activities of hepatic detoxification enzymes, which partly explains differential tolerance to PSCs (Haley et al., 2008). However, the experienced and naïve populations also harbor unique foregut microbial communities (Kohl and Dearing, 2012). This observation led us to hypothesize that the microbiota of experienced woodrats facilitates ingestion of dietary toxins.

Results and Discussion

To test whether the woodrat microbiota is responsive to PSCs, I conducted metagenomic sequencing on the foregut contents of animals from the experienced population that were fed either a toxin-free diet of rabbit chow, or one amended with 2% creosote resin by dry weight. PSC ingestion definitively shaped the microbiota at the

gene level; creosote-fed animals harbored a microbiota with a higher abundances of genes associated with the metabolism of aromatic compounds (Fig. 8.1). Specifically, the microbiota of creosote-fed animals exhibited a significantly higher abundance of genes encoding aryl-alcohol dehydrogenases (Fig. 8.2), which catalyze the first step in degrading aromatic alcohols, and are therefore expected to play a role in degrading NDGA. Metagenomic analysis revealed 58 unique candidate aryl-alcohol dehydrogenase (AAD) genes that resemble those isolated from bacteria in the family Lactobacillaceae (Fig. 8.3).

In order to remove the contribution of the microbiota, woodrats from the experienced (Mojave) population were treated with neomycin, a broad spectrum antibiotic that is poorly absorbed across the gut tissue and reduces gut bacterial density by ~90% (Vijay-Kumar et al., 2010). The resulting animals were then fed either a control diet or one supplemented with 2% creosote resin. Animals were removed from the trial when they lost more than 10% of their original body mass. A diet of 2% creosote resin represents a low dose of PSCs for experienced animals, and is tolerable even for naïve animals (Mangione et al., 2000). Antibiotic treatment (AB) significantly altered the microbial community composition and decreased microbial diversity by roughly 50% (Fig. 8.4). Antibiotics did not impair the ability of woodrats to feed on a control diet and maintain body mass (Bonferonni-corrected P-values; No AB, control diet vs AB, control diet, $P = 0.20$; Fig. 8.5). However, antibiotic-treated animals fed the creosote diet could not ingest enough food to maintain mass (No AB, creosote diet vs. AB, creosote diet, $P < 0.001$; AB, control diet vs. AB, creosote diet, $P = 0.009$; Fig. 8.5). No individual given the AB treatment and fed creosote PSCs lasted more than 13 days in the trial. For comparison, naïve individuals can ingest a diet of 2% creosote resin for weeks in the

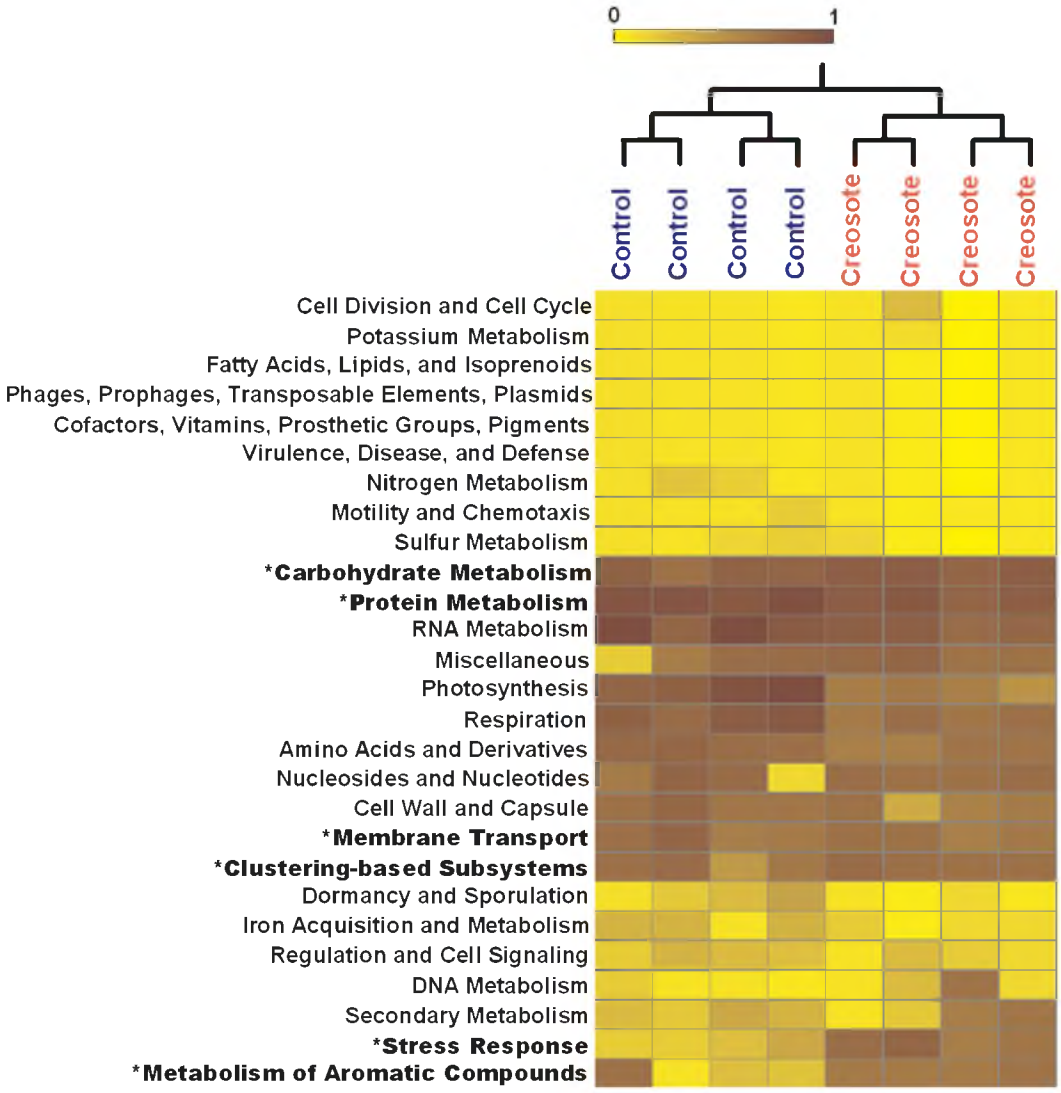

Fig. 8.1: Heat map showing normalized abundances of genes in various functional categories in the foregut metagenomes of animals fed a control diet, or one with 2% creosote resin. Each column represents an individual animal, and columns are clustered according to similarity in functional profiles. Each row represents a functional category. Those in bold with an asterisk are significantly more abundant in animals fed creosote resin.

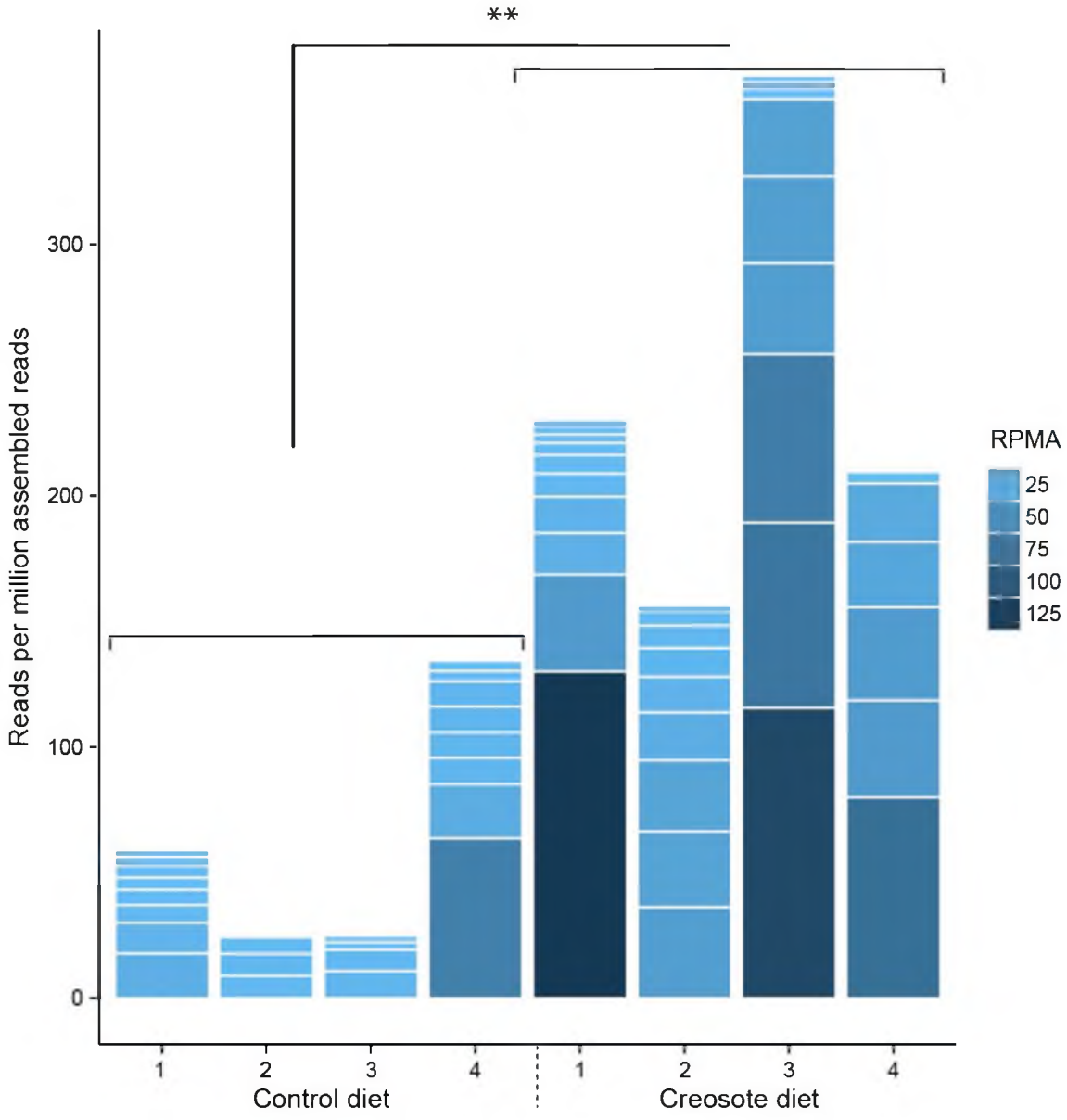

Fig. 8.2: Normalized reads per million assembled reads (RPMA) of candidate aryl-alcohol dehydrogenase ORFs. Unique ORFs are different portions of vertical bars. Creosote fed animals had a significantly higher abundance of these sequences ($P = 0.01$).

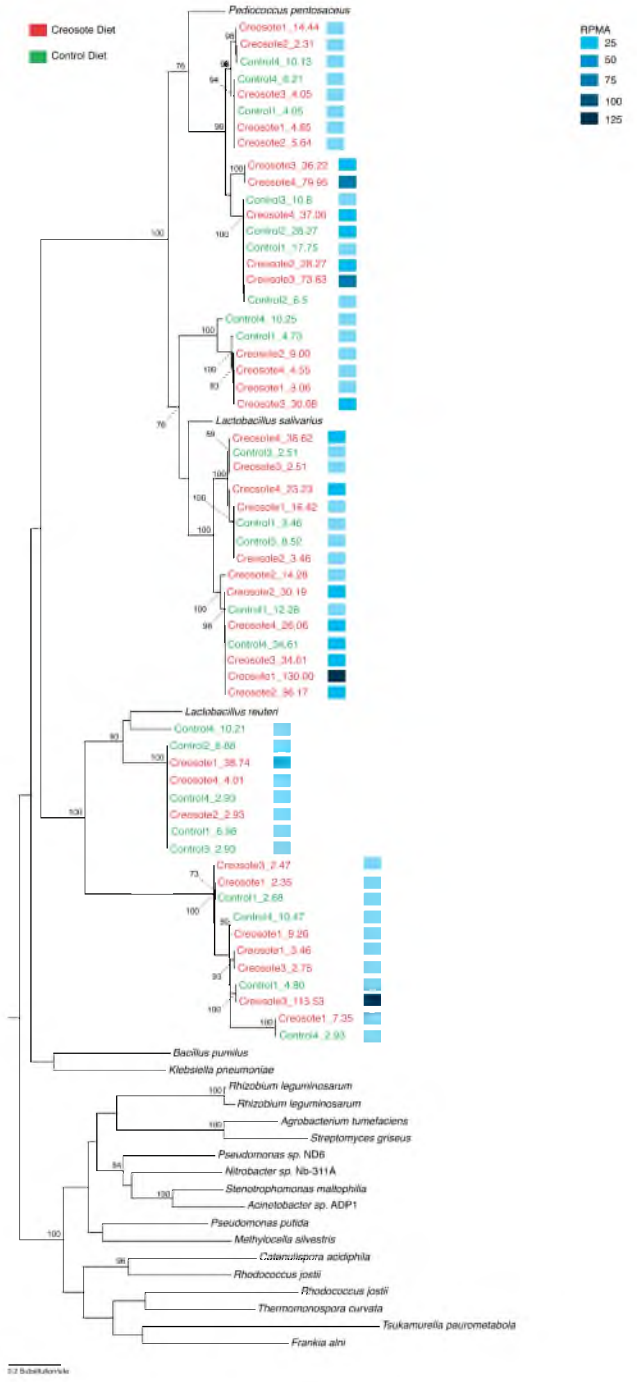

Fig. 8.3: Phylogeny of protein sequences from candidate aryl-alcohol dehydroganse genes found in the woodrat metagenome, and those in the SEED database. Tree was trimmed to remove very similar sequences from the database. Red nodes represent those ORFs found in creosote fed animals, blue nodes represent those found in control fed animals. The box next to each node corresponds to the reads per million assembled reads (RPMA) of the candidate sequence.

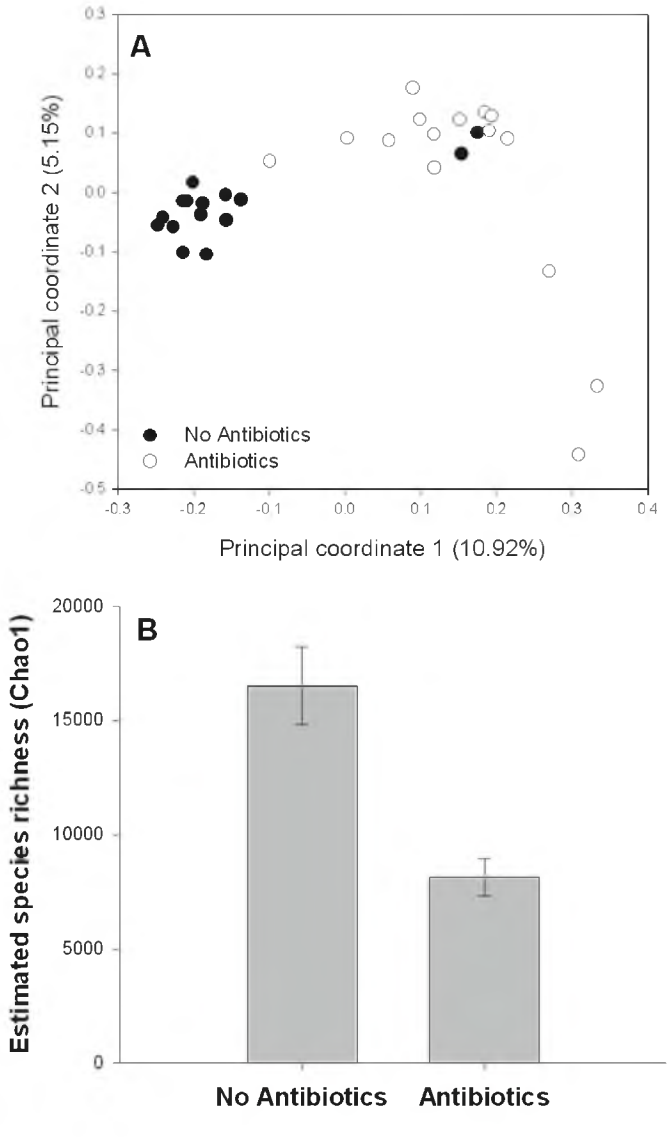

Fig. 8.4: Effect of antibiotics on gut microbiota. (A) Principal coordinates analysis of unweighted UniFrac distances of fecal 16S rRNA inventories from control and antibiotic treated animals. ANOSIM analysis revealed that antibiotics significantly altered microbial communities representation (100 permutations, R = 0.447, P = 0.01). (B) Estimated species richness of fecal communities, as determined by Chao1. Twenty permutations of 16,270 sequences per sample were generated and averaged within samples. Treatment means were compared using a paired t-test. Antibiotics significantly decreased estimated species richness ($P < 0.0001$).

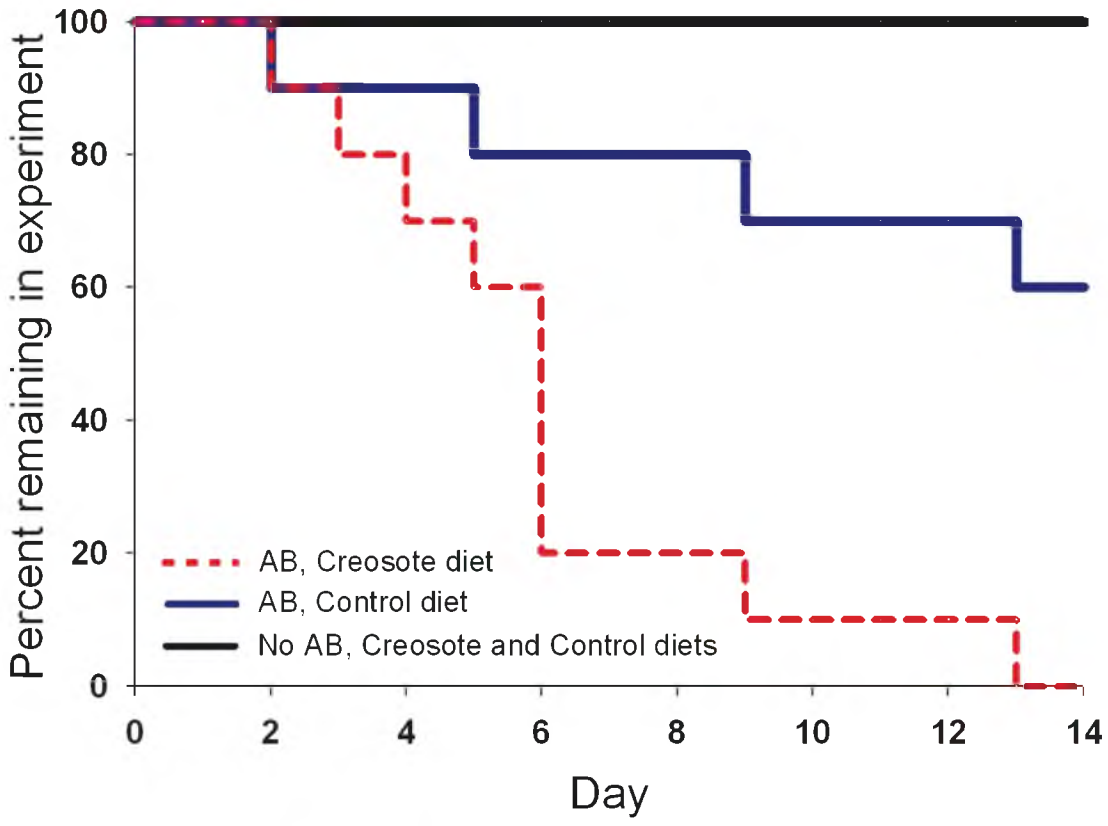

Fig 8.5: Persistence analysis of experienced animals fed either a control diet, or one containing 2% creosote resin, either with or without antibiotics (AB). Sample sizes (N) for each group are as follows: AB, creosote diet: 10; AB, control diet: 10; No AB, control diet: 7; No AB, creosote diet: 8

laboratory with no decline in mass (Mangione et al., 2000). Thus, depletion of the microbiota effectively removed 17,000 years of ecological and evolutionary experience with creosote PSCs.

To determine whether the tolerance to PSCs of the experienced population could be conferred to the naïve population, I conducted fecal transplants from experienced woodrat donors into naïve recipients. As a control, a group of naïve recipients received feces from other naïve individuals. Because woodrats naturally cache their feces and are coprophagic (Kenagy and Hoyt, 1980), this experiment mimics an ecologically relevant route of transmission. Feces were ground and integrated into the food for several days, after which the concentration of creosote resin in the diet was increased every few days. Gradually increasing the concentrations of toxins allowed us to determine upper thresholds for animals. Prior to the transplant, both groups exhibited similar microbial communities (Pre-experiment animals: ANOSIM, $P = 0.67$; Fig. 8.6A). Following the transplant, experienced recipients maintained a microbiota that closely resembled their experienced donors, and differed from control recipients (ANOSIM, $P = 0.001$; Fig 8.6A). When fed a diet lacking creosote resin, there was no difference in body mass between groups (Days 0-3; Fig. 8.6B). However, when creosote resin was added to the diet, the experienced recipients maintained a higher body mass ($P = 0.008$; Fig. 8.6B). Moreover, the experienced recipients were able to consume higher concentrations of creosote resin and persist in the trial significantly longer than the control group ($P = 0.038$; Fig. 8.6C). Thus, transplantation of creosote-experienced microbiota substantially increased the tolerance of naïve animals to dietary toxins.

Although I observed a change in body mass between experienced and control recipients when fed a 2% creosote resin, I did not observe differences in food intake or dry matter

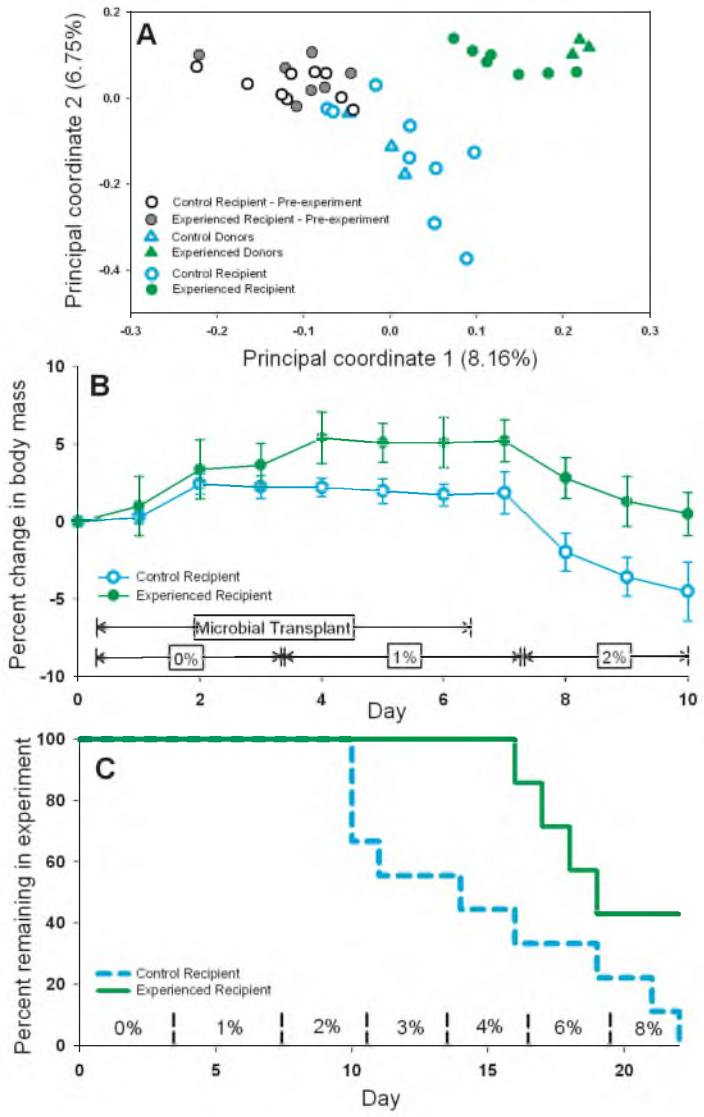

Fig. 8.6: Microbial transplants improve performance on toxic diets. (A) Principal coordinate analysis of unweighted UniFrac distances generated from 16S rRNA microbial inventories of feces from donors and recipients. Black and grey circles represent animals before microbial transplant. Colored circles represent microbial communities on day 10. (B) Percent change in body mass (mean ± SEM) of control and experienced transplant groups over time. Labels above the x-axis denote when the microbial transplant was conducted, and the percentage of creosote resin in the diet. (C) Persistence analysis of control and experienced transplant groups throughout the trial. Labels on the x-axis correspond to the percentage of creosote resin in the diet. Sample sizes (N) for all panels are as follows: control transplant: 9; experienced transplant: 7.

digestibility between groups. These results are consistent with earlier studies comparing naïve and experienced woodrats on a 2% resin diet (Mangione et al., 2000). Thus, the maintenance of body mass in the experienced recipients fed low-resin diets was likely due to lower costs of hepatic detoxification, which can be as high as 50% of basal metabolic costs (Sorensen et al., 2005). Indeed, the experienced recipients produced less acidic urine (Quadratic mixed effects model, Donor effect: $P = 0.035$; Fig. 8.7A), indicating decreased hepatic detoxification (Foley et al., 1995; Mangione et al., 2001). The experienced recipients produced urine with unique metabolite signatures compared to the control group fed a 1% creosote diet (Fig. 8.7B). This difference was not observed in animals fed a creosote-free diet following the end of the trial. I also identified several urinary metabolites that were produced only by experienced or control recipients fed creosote (Fig. 8.7C-D). Taken together, these results suggest that the inoculation of woodrats with an experienced microbiota altered detoxification routes and facilitated ingestion of PSCs. It will be interesting to investigate the relative contributions of microbial detoxification and metabolic cross-talk between the microbiota and the liver.

This work shows that gut microbes play an important role in facilitating the ingestion of dietary toxins by wild herbivores. The loss of these microbes clearly limits their ability to feed on toxic plants. This may be of concern for animals exposed to antibiotic pollution (Kemper, 2008) or bred through captive rearing programs (Redford et al., 2012). Likewise, the gain of such microbes can facilitate the consumption of toxic plants. Microbial probiotics are of interest to farmers aiming to increase the feed efficiency of livestock (Krehbiel et al., 2003), and detoxifying microbes might provide a means to engineer livestock that can be reared in an environment containing toxic plants. Moreover, symbiotic interactions such as those reported in this study may have

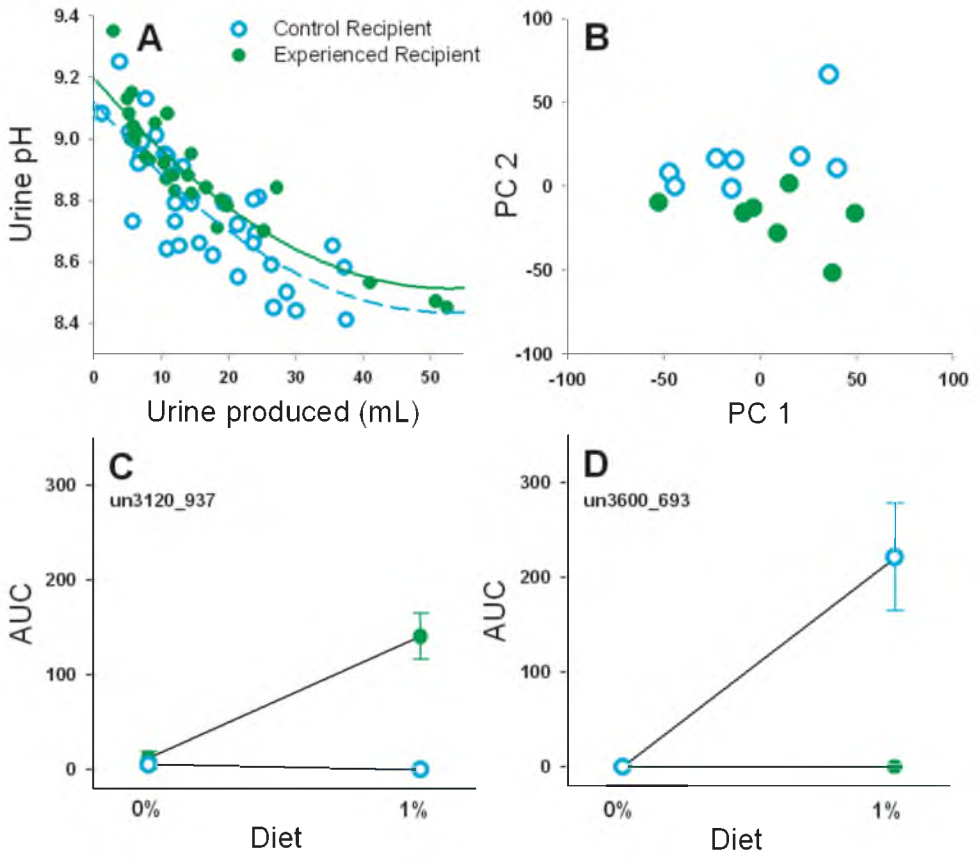

Fig. 8.7: Microbial transplants alter detoxification routes. (A) Urine pH as a function of urine produced. (B) Principal component analysis of over 800 urinary metabolites from animals fed a 1% creosote resin diet. (C and D) Examples of two unknown urinary metabolites produced from either experienced (C) or control (D) groups fed a 1% creosote resin diet. Numbers under the panel label refer to the retention index of the metabolite. Metabolites were not detectable when animals were returned to a 0% resin diet at the end of the trial. The amounts of each metabolite detected are expressed as area under the curve (AUC; mean ± SEM). Sample sizes (N) for all panels are as follows: control transplant: 9; experienced transplant: 7.

influenced the foraging ecology and evolution of many herbivorous mammalian species, thus greatly influencing ecosystem structure worldwide. In the future, wild herbivores may be faced with novel and more potent plant toxins due to changes in land use, introduction of exotic species, or global climate change (Verhoeven et al., 2009; Dearing, 2013). Although past rates of niche evolution lag far behind the pace at which humans are expected to alter ecosystems (Quintero and Wiens, 2013), microbial detoxification may represent a mechanism for accelerated adaptation and niche expansion in herbivores.

Methods

Animals and Diets

All animals were collected from the wild using Sherman live traps. Woodrats for the metagenome experiment were collected from Lytle Ranch, Washington Co., UT (37°07'N, 114°00'W) in October, 2010. Animals for the antibiotic treatment experiment were also collected from Lytle Ranch in July 2011. Experienced donors for the microbial transplant experiment were collected from Lytle Ranch in May, 2012. Naïve donors and recipients were collected from White Rocks, Tooele Co., UT (40°19′N, 112°54′W) in November 2012. All woodrats were transported to the University of Utah Department of Biology Animal Facility and housed in individual cages (48 × 27 × 20 cm) under a 12:12-hr light:dark cycle, with 28°C ambient temperature and 20% humidity.

Prior to experimentation, animals were maintained on a diet of high-fiber rabbit chow (Harland Teklad formula 2031). During experimentation, animals were fed the same chow except in a powdered form to prevent caching of food. To prepare diet treatments containing creosote resin, creosote leaves were collected from trapping sites and frozen at -20°C prior to resin extraction. I performed surface extractions by soaking

leaves in acetone (1:6, wet leaf mass:volume solvent) for 45 min. Solvent and resin were filtered (Whatman filter paper grade 1) to remove large particles and evaporated using a rotovap until the resin was highly viscous, at which point it was transferred to a vacuum pump for 48 hr to remove any remaining acetone. Extracted resin was stored at -20°C prior to use.

Creosote diet treatments were prepared by dissolving the appropriate amount of resin in a volume of acetone equal to 25% of the dry weight of ground rabbit chow to which it was added. Control diet (0%) was prepared by adding an identical ratio of acetone, without creosote resin. Acetone was evaporated from all diets in a fume hood, and complete evaporation was confirmed gravimetrically.

Metagenomics

Four individuals from the experienced population served as control animals and were fed powdered rabbit chow in cages for 8 days. Another four individuals were fed the control diet for 3 days, followed by the same diet with increasing amounts of creosote resin (1 and 2% creosote resin for 2 and 3 days, respectively). Following diet treatments, animals were euthanized under CO_2, and immediately dissected. Contents of the foregut were removed and frozen at -80°C prior to DNA isolation.

Foregut contents were thawed on ice and a small amount (~25 mg) was incubated with 180 μL enzymatic lysis buffer (20 mM Tris·Cl; pH 8.0, 2 mM sodium EDTA, 1.2% Triton X-100 and 20 mg/ml lysozyme) at 37°C for 30 min. DNA was extracted using a QIAGEN DNeasy Blood and Tissue Kit.

Total genomic DNA was sequenced on an Illumina HiSeq 2000 platform at the University of Utah Microarray and Genomic Analysis Core Facility to yield 100 base

paired-end reads.

Unassembled metagenomic data were compared between treatment groups using MG-RAST (Meyer et al., 2008). Sequences were screened against the genome of *Arabidopsis thaliana* to remove potential contamination from the plant-based diet. The reads were then filtered using dynamic trimming with a quality threshold of 15, such that any sequences with more than 5 low quality bases were removed. Sequences were annotated with the SEED Subsystems (Overbeek et al., 2005) with the following thresholds: (1) e-values less than 1e-5; (2) a minimum percent identity to database sequences of 60%; and (3) a minimum alignment length of 30 bases. Abundances of functional categories were normalized, and a heatmap and dendrogram were generated using a clustering algorithm within MG-RAST. Abundances of functional categories were also compared between control- and creosote-fed animals using t-tests.

The metagenomes were also assembled using IDBA-UD (Peng et al., 2012) with standard parameters, including "--pre_correction." I searched the metagenomes for aryl-alcohol dehydrogenase genes by creating a database of 42 aryl-alcohol dehydrogenase protein sequences from the SEED database, and searching translated metagenomic sequences using TBLASTN. Hits were parsed using a minimum e-value of 1e-70 and a minimum translated length of 300 amino acids. Normalized 'reads per million assembled reads' were calculated for each resulting candidate gene, and compared between control- and creosote-fed animals using a one-way ANOVA. Phylogenetic placement of candidate AAD genes was determined by aligning candidate AAD genes using MUSCLE (Whelan and Goldman, 2001), and creating a phylogenetic tree of select sequences from the SEED database and translated metagenomic contigs using PhyML v3.0 (Guindon et al., 2010) with the WAG amino-acid substitution model (Whelan and Goldman, 2001) with 25

random starting trees and 100 bootstrap replicates.

Antibiotic Treatment

The antibiotic experiment was conducted within 1 week of animal capture. Ten animals were fed powdered rabbit chow, and another ten animals were fed rabbit chow supplemented with 2% extracted creosote resin. Within each diet treatment, five animals were given neomycin in their water (0.5 g/L) with sucralose (2.5% w/v) to encourage drinking. Five animals were given water containing only sucralose as a control. Body mass was monitored for two weeks, and animals were removed from the trial if they lost more than 10% of their original body mass. Following the trial, animals were given at least two weeks to recover body mass. Afterwards, antibiotic treatments were switched and the trial was repeated. Most animals recovered their microbiota relatively quickly (Fig. 8.4). Some animals could not be used in the second trial due to excessive weight loss, and the resulting sample sizes in each group were: No AB, control diet: 7; No AB, creosote diet: 8; AB, control diet: 10; AB, creosote diet: 10. Persistence curves were compared using a Log-Rank Kaplan-Meier survival analysis and adjusted for multiple comparisons using the Bonferroni method.

Microbial Transplant

Naïve woodrats were collected from the wild and allowed to acclimate to captivity for 10 days. Donors (experienced and naïve) were kept in metabolic cages and fed powdered rabbit chow containing 2% creosote resin to prime their microbiota. I collected and ground feces from these animals daily. Ground feces (15% w/w) from either experienced and naïve donors was added to the food of recipients for 6 days. The

experienced recipients (N=7) received feces from experienced individuals, while the control recipients (N=9) received feces from other naïve individuals. All recipient animals were kept in metabolic cages and fed an increasing amount of creosote resin in their diet (0, 1, 2, 3, 4, 6, 8%, increasing every 3 days). I measured food intake and body mass daily. Animals were removed from the trial if they lost more than 10% of their original body mass. Animals were then fed a 0% diet for at least 4 days to facilitate recovery. Persistence curves were compared using a Log-Rank Kaplan-Meier survival analysis. Urine and feces were collected daily. Urine pH was measured with an Omega Soil pH electrode (PHH-200). Feces were dried at 45°C overnight and weighed. I calculated dry matter digestibility as the [(grams food intake – grams feces output)]/grams food intake. Body mass, food intake, and dry matter digestibility were all compared using repeated measures ANOVA.

16S rRNA Inventories

I collected feces for microbial analysis from both the antibiotic and transplant experiments. For the antibiotic trial, I collected feces on the final day of each treatment. For the transplant experiment, I collected feces before the trial, as well as on the last day of 2% diet, which was 4 days after the conclusion of the transplant. Whole DNA was extracted from all feces using a QIAamp DNA Stool Mini Kit (Qiagen). A previously established technique was used to amplify the V4 region of the 16S rRNA gene with primers 515F and 806R (Caporaso et al., 2011). The reverse primer also contained a 12 base barcode sequence, allowed for pooling of samples. PCR reactions were conducted in triplicate and resulting products were pooled within a sample. DNA was quantified using Invitrogen's PicoGreen and a plate reader and cleaned using the UltraClean PCR Clean-

Up Kit (MoBIO). Amplicons were sequenced on the Illumina MiSeq platform using previously described techniques (Caporaso et al., 2012). Sequences were analyzed using the QIIME software package (Caporaso et al., 2010). Sequences underwent standard quality control and were split in to libraries using default parameters in QIIME. Sequences were grouped into operational taxonomic units (OTUs) using UCLUST (Edgar, 2010) with a minimum sequence identity of 97%. The most abundant sequences within each OTU were designated as a "representative sequence," and aligned against the Greengenes core set (DeSantis et al., 2006) using PyNAST (Caporaso et al., 2009) with default parameters set by QIIME. A PH Lane mask supplied by QIIME was used to remove hypervariable regions from aligned sequences. FastTree (Price et al., 2009) was used to create a phylogenetic tree of representative sequences. OTUs were classified using the Ribosomal Database Project (RDP) classifier with a standard minimum support threshold of 80% (Wang et al., 2007). Sequences identified as chloroplasts or mitochondria were removed from the analysis.

I calculated estimated species richness, or Chao1, which estimates the asymptote on a species accumulation curve. I compared community memberships (presence or absence of lineages, and not their relative abundances) of treatment groups. I also compared diversity between samples (β diversity) by calculating unweighted UniFrac scores, which measures diversity shared between samples by determining the fraction of branch length shared between two samples in the phylogenetic tree created from all representative sequences. I then conducted Principal coordinates analysis (PCoA) on unweighted UniFrac scores to investigate similarities. Similarities were tested using the ANOSIM function within QIIME.

Urine Extraction and GC-MS Analysis

I conducted metabolomic analysis on urine samples from the transplant experiment on the third day of the 1% diet, as well as on day 4 of the 0% diet recovery diet at the end of the trial. This collection schedule allowed us to conduct metabolomics on a 0% diet after the transplant. A methanol extraction was used to remove protein from urine prior to analysis. In brief, 900 µL of -20°C 90% methanol (aq.) was added to 40 µL of the individual tubes containing the cell pellet to give a final concentration of 80% methanol. The samples were incubated for 1 hr at -20°C followed by centrifugation at 30,000 x g for 10 minutes using a rotor chilled to -20°C. The supernatant containing the extracted metabolites was then transferred to fresh disposable tubes and completely dried *en vacuo*.

All GC-MS analysis was performed with a Waters GCT Premier mass spectrometer fitted with an Agilent 6890 gas chromatograph and a Gerstel MPS2 autosampler. Dried samples were suspended in 40 µL of a 40 mg/mL O-methoxylamine hydrochloride (MOX) in pyridine and incubated for 1 hr at 30°C; 25 µL of this solution was added to autosampler vials. Ten microliters of N-methyl-N trimethylsilyltrifluoracetamide (MSTFA) was added via the autosampler and incubated for 60 min at 37°C with shaking. After incubation 3 µL of a fatty acid methyl ester standard solution was added via the autosampler then 1 µL of the prepared sample was injected to the gas chromatograph inlet in the split mode with the inlet temperature held at 250°C. A 5:1 split ratio was used for cell culture analysis and a 50:1 split was used for urine analysis. The gas chromatograph had an initial temperature of 95°C for 1 min followed by a 40°C/min ramp to 110°C and a hold time of 2 min. This process was followed by a second 5°C/min ramp to 250°C, a third ramp to 350°C, then a final hold

time of 3 min. A 30 m Phenomex ZB5-5 MSi column with a 5 m long guard column was employed for chromatographic separation. Helium was used as the carrier gas at 1 mL/min.

Analysis of GC-MS data

Data were collected using MassLynx 4.1 software (Waters). A two-step process was employed for data analysis, a targeted followed by nontargeted analysis. For the targeted approach, known metabolites were identified and their peak area was recorded using QuanLynx. These data were transferred to an Excel spread sheet (Microsoft, Redmond WA). For the nontargeted approach peak picking and analysis was performed using MarkerLynx. Principle component analysis (PCA) and partial least squares-discriminate analysis (PLS-DA) was performed using SIMCA-P 12.0 (Umetrics, Kinellon, NJ). Potential metabolite biomarkers were further investigated by manually recording the peak area into the original Excel file followed by performing t-tests. Potential metabolite biomarkers were further investigated by manually recording the peak area into the original Excel file followed by performing t-tests. For metabolites that were found at high concentrations in the 10:1 analysis, particularly proline, aspartic acid, and glutamic acid, I used the 100:1 data set to record accurate data. These data were normalized for extraction efficiency and analytical variation by mean centering the area of D4-succinate.

Data Deposition

Foregut metagenomes have been deposited in MG-RAST. Microbial inventories of the antibiotic and transplant experiments have been deposited in the Sequence Read

Archive (SRA) under accession code SRP027399.

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APPENDIX A

DIVERSITY AND FUNCTION OF THE AVIAN GUT MICROBIOTA

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REVIEW

Diversity and function of the avian gut microbiota

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Abstract The intestinal microbiota have now been shown to largely affect host health through various functional roles in terms of nutrition, immunity, and other physiological systems. However, the majority of these studies have been carried out in mammalian hosts, which differ in their physiological traits from other taxa. For example, birds possess several unique life history traits, such as hatching from eggs, which may alter the interactions with and transmission of intestinal microbes compared to most mammals. This review covers the diversity of microbial taxa hosted by birds. It also discusses how avian microbial communities strongly influence nutrition, immune function, and processing of toxins in avian hosts, in manners similar to and different from mammalian systems. Finally, areas demanding further research are identified, along with descriptions of existing techniques that could be employed to answer these questions.

Keywords Avian hosts · Intestinal microbes · Symbiosis

Introduction

Vertebrate animals maintain complex and intimate associations with a diverse community of microbes residing in their intestinal tracts (Ley et al. 2008b). Previously, it was believed that the main benefit of hosting these microbes was to be able to utilize novel food sources, such as cellulose. However, recent research has revealed that these microbes play a large role in many aspects of an animal's physiology, including proper development of intestinal morphology and digestive function, as well as immune function (Leser and Mølbak 2009). Though the diversity of microbes, as well as their roles and importance in mammalian physiology have been elucidated, the biological significance of intestinal microbes in birds remains largely unknown.

Birds represent interesting study systems in which to investigate the roles of intestinal microbes, because they have extremely complex and unique diets, physiological traits, and developmental strategies. Additionally, the capacity for flight has been a strong selective pressure on many aspects of their physiology, perhaps changing the nature of their intestinal fauna. Many studies on microbial community function have been conducted on domestic bird species, which allow us to infer the biological role of intestinal microbes in wild birds. Taken together, results suggest that intestinal microbes have large effects on the nutrition, immune function, and development of their avian hosts. This review examines and compares microbial relationships between birds and mammals in order to highlight gaps in knowledge and identify experimental questions for the future.

Diversity of avian intestinal microbes

There have been several efforts to characterize the microbial diversity of the avian gut; however, the majority of these studies use selective, culture-based techniques to investigate microbial species of interest, and largely focus on identifying potentially pathogenic microbes (Craven et al. 2000; Gaukler et al. 2009). These techniques are not ideal, especially for the study of mutualistic microbial diversity, as it is estimated that 99% of microbial species

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cannot be cultured under laboratory conditions (Rappe and Giovannoni 2003). Fortunately, advances in culture-independent methods have allowed for more complete inventories of the intestinal microbial community.

The ideal method to conduct a microbial inventory is through 16S rDNA sequence analysis, which has only been carried out on the gut contents of eight avian taxa (Table 1). In contrast, a single study conducted inventories on 60 mammalian species (Ley et al. 2008a). For the most part, microbial communities at higher taxonomic levels are very similar between birds and mammals; most studies show 2 phyla, Firmicutes and Bacteroidetes, as dominant out of 75 known microbial phyla. This dominance is not surprising: it is believed that the common ancestor of amniotes (reptiles, birds, and mammals) maintained a microbial community mostly comprising Firmicutes and Bacteroidetes (Costello et al. 2010). At this point, comparing the abundance of microbes across studies is not possible due to the limited number of species, varied sources (feces vs. crop), and different techniques. It would be useful to adopt standardized approaches for future inventories of avian species (see Ley et al. 2008a for an example) to investigate the effects of host diet, taxonomy, and gut anatomy on intestinal microbial communities.

All phyla detected from birds using 16S sequencing have also been identified in mammalian microbial communities, suggesting that birds may not harbor unique microbial phyla. However, a recent study on the hoatzin crop, using DNA microarray techniques, documented for the first time the presence of phyla such as Aquificae, Coprothermobacteria, Thermodesulfobacteria and Caldithrix in a vertebrate gut system (Godoy-Vitorino et al. 2010). These techniques will need to be replicated on mammalian gut contents to determine if these phyla are unique to the hoatzin crop. At lower taxonomic levels, there are genera and species unique to birds. For example, 16S rDNA inventory of the hoatzin revealed that 94% of the phylotypes present represented completely novel microbial species and genera (Godoy-Vitorino et al. 2008). Additionally, researchers have found host-specific species in both the gull (*Larus* spp.) and Canada goose (*Branta canadensis*) (Lu et al. 2008, 2009).

In addition to bacteria, members of the domain Archaea are present in the intestinal communities of many birds. However, not all studies listed in Table 1 investigated the presence of Archaea. Targeted investigations have since shown the presence of Archaea in the hoatzin crop (Wright et al. 2009) and chicken cecum (Saengkerdsub et al. 2007). These isolates are often methanogens, which are important for removing the excess hydrogen ions produced by fermentation. The presence and functional roles of Archaea are often overlooked in basic microbial inventories (Baker et al. 2006), but should be investigated in avian systems.

Interactions between symbiotic microbes and avian physiology

In mammals, gut microbial communities are determined by host taxonomy, diet, and gut anatomy (Ley et al. 2008a), and the functions of these communities can be predicted based on 16S rDNA sequence inventories (Muegge et al. 2011). However, due to variability in gene content between even closely related strains of microbes (Nelson et al. 2010), as well as potential novelty in understudied avian systems, it may be difficult at this stage to assign putative functions to microbial populations based on avian 16S inventories alone. There have been several studies investigating the physiological functions and host interactions of microbes in wild birds and many well-developed experiments using germ-free chickens to investigate impacts on the hosts themselves. Results have elucidated that intestinal microbes play large roles in the nutrition, immune function, and processing of toxins of avian hosts; these results are described in greater detail below.

Nutrition

In many mammals, microbes aid in the host's ability to utilize plant polysaccharides, such as cellulose, as energy sources (Dehority 1997). However, in birds, the presence of fibrolytic microbes depends greatly on gut location and host phylogeny. Like mammals, some birds such as the hoatzin (Grajal et al. 1989) and ostrich (Matsui et al. 2010) maintain large, fibrolytic fermentation chambers. In these species, fermentation end products can supply large proportions of their total energy budgets (75% in the ostrich and 80% in ruminant mammals, compared to only 10–20% in other domestic fowl; Jozefiak et al. 2004). Cellulolytic microbes have also been isolated from the pigeon crop (Shetty et al. 1990), but due to low residence time in this chamber extensive fiber metabolism is not likely.

Microbes residing in the intestines of other bird species, however, are simply saccharolytic rather than cellulolytic, and thus only aid the avian host in utilization of substrates which it could otherwise digest itself (Vispo and Karasov 1997). Amylase activity, which is presumably microbial due to limited activity of salivary amylase in birds (Stevens and Hume 2004), has been detected in the crops of the chicken and turkey (Bolton 1965; Pinchasov and Noy 1994). Additionally, amylolytic, but not cellulolytic, microbes have been isolated from the crop of the green-rumped parrotlet (*Forpus passerinus*) (Pacheco et al. 2004). The ceca of most Galliformes are not thought to be extensive fibrolytic chambers, but do contain saccharolytic bacteria (Vispo and Karasov 1997). These microbes may conduct microbial fermentation of starches and simple sugars, which provides relatively less energy to the host

Table 1 Abundances of microbial taxa from the gut contents of previously studied birds and mammals

Species	Adelie Penguin *Pygoscelis adeliae*	Gull *Larus spp.*	Canada Goose *Branta canadensis*	Ostrich *Struthio camelus*	Turkey *Meleagris gallopavo*	Chicken *Gallus gallus*	Hoatzin *Opisthocomus hoazin*	Parrots Various species	Mouse *Mus musculus*	Cow *Bos taurus*	Horse *Equus ferus*
Source	Feces	Feces	Feces	Cecum	Cecum	Cecum	Crop	Cloaca	Cecum	Rumen	Hindgut
Microbial phyla	% of Community										
Firmicutes	39.2	54.6	71.6	50.9	32.3	70	66.3	72.9	58.9	22.3	36.8
Bacteroidetes	14.7	1.1	10.1	39.4	54.2	1.9	29.9	0.2	24.1	45.2	47.4
Actinobacteria	32.3	6.4	7.0	–	<0.1	4.9	0.8	12.0	–	–	–
Proteobacteria	9.8	23	10.4	–	3.4	21.5	1.6	14.9	2.6	26.9	–
Tenericutes	3.9	8.9	0.2	–	–	<0.1	0.2	–	13.9	–	–
Fibrobacteres	–	–	–	6.5	–	–	–	–	–	3.6	–
Deferribacteres	–	–	–	–	2.6	–	–	–	–	–	–
Spirochaetes	–	1.1	–	1	–	–	0.2	–	–	0.5	3.5
Fusobacteria	–	0.7	–	–	–	–	–	–	–	–	–
Planctomycetes	–	0.4	–	–	–	–	–	–	–	–	–
Cyanobacteria	–	0.4	–	–	–	–	–	–	–	–	–
Verrucomicrobia	–	–	–	0.3	–	–	<0.1	–	–	–	8.8
Synergistetes	–	–	–	–	–	–	0.2	–	–	–	–
TM7	–	–	0.1	–	–	–	<0.1	–	–	–	–
Lentisphaerae	–	–	–	–	–	–	<0.1	–	–	–	–
Archaea	–	–	–	1.9	–	–	–	–	–	1.5	3.5
Unknown	–	3.4	0.5	–	7.3	1.7	0.6	–	–	–	–
Source	(Banks et al. 2009)	(Lu et al. 2008)	(Lu et al. 2009)	(Matsui et al. 2010)	(Scupham et al. 2008)	(Zhu et al. 2002)	(Godoy-Vitorino et al. 2008)	(Xenoulis et al. 2010)	(Kibe et al. 2004)	(An et al. 2005)	(Yamano et al. 2008)

than hydrolysis by endogenous enzymes (Stevens and Hume 2004), but may still increase absolute energy extraction for the host.

Though present, these cellulolytic and saccharolytic microbes make up just a small proportion of the avian microbial community. Rather, microbes capable of degrading uric acid are much more abundant (Mead 1989). Uric acid is the main product of nitrogen metabolism in birds and can be moved into the lower intestinal tract and ceca through retrograde peristalsis. Here, it can be converted to microbially synthesized amino acids that can be reabsorbed by the host (Vispo and Karasov 1997). The process of uric acid metabolism by microbes is thought to be especially important for conserving nitrogen, especially in species with low protein diets. Indeed, uric acid metabolizing microbes have been isolated from the intestinal tract of the chicken, turkey, guinea fowl, duck, pheasant, and hummingbird (Barnes 1972; Preest et al. 2003). It is especially remarkable that these microbes have been isolated from the hummingbird because hummingbirds lack ceca and have extremely fast digesta throughput (Stevens and Hume 2004), which could make colonization by microbes challenging. However, whether this metabolic capability represents a significant contribution to the nitrogen economy of avian hosts remains to be explored.

Microbes are also known to increase nutrient absorption in mammals (Tennant et al. 1971). For example, gnotobiotic mice colonized with a single species of microbe (*Bacteroides thetaiotaomicron*) exhibited 2.6 times higher intestinal expression of the sodium-glucose transporter protein (SLGT-1) compared to germ-free mice (Hooper et al. 2001). However, colonization of the gut also greatly increases the integrity of the epithelial wall through upregulation of many cross-bridging proteins, presumably to decrease invasion by pathogenic microbes or absorption of endotoxins (Hooper et al. 2001). This epithelial fortification may actually inhibit nutrient absorption in avian species. Due to the selective pressure of flight, birds have decreased intestinal surface area compared to non-flying mammals, and rely more heavily on paracellular absorption, the process by which water-soluble nutrients are transferred between epithelial cells (Caviedes-Vidal et al. 2007). Studies with germ-free chickens have indeed shown that colonization by microbes decreases total absorption of glucose and vitamins (Ford and Coates 1971). The mechanisms of trade-offs between microbial colonization and nutrient absorption in birds remain to be explored.

Immune function

Intestinal microbes are known to greatly influence the cost, development, and effectiveness of mucosal and systemic immune responses in mammalian systems (Macpherson and Harris 2004). This trade-off is well evidenced by the fact that germ-free mammals overall have depressed immune functions. They have decreased cytokine production, systemic immunoglobulin levels, intraepithelial lymphocyte counts, and relative amounts of gut-associated lymphoid tissue (GALT). As a result, these animals are more susceptible to infection (O'Hara and Shanahan 2006). Interactions between intestinal microbes and the immune systems of avian hosts are often assumed to be similar to mammals, yet they have been largely undescribed (Brisbin et al. 2008). The relative lack of information on microbe–host immunity interactions is especially remarkable given that birds have many unique and interesting characteristics of their immune systems.

One unique aspect of the avian immune system is the bursa of Fabricius, the primary site of B cell development. Mammalian B cells develop in the bone marrow, far removed from the intestinal tract and commensal microbes. While sampling and transport of microbial antigens from the intestinal lumen have been shown in mammalian systems (Owen et al. 1986), the bursa of Fabricius is a diverticulum of the intestinal tract itself and is known to be colonized by microbes shortly after hatching (Kimura et al. 1986). These microbes may act as antigens themselves or induce production of cytokines, increasing the proliferation and maturation of bursal B cells (Ratcliffe 2006). When the bursal duct is experimentally ligated prior to hatching, chickens exhibit lower natural antibody production, suggesting that gut microbes can have systemic effects on immunity through this structure (Ekino et al. 1985). Likewise, infusion of killed bacterial antigens into the ligated bursal lumen recovered natural antibody production to greater than that of control chickens (Ekino et al. 1985). Further research must be done to determine if gut microbes play an increased role in B cell development in birds compared to mammals due to their intimate association with the bursa of Fabricius.

Birds also differ from mammals in several aspects of cell-mediated immunity. For example, birds have fewer gene families of the T-cell receptor gene (Lahti et al. 1991), which may influence the diversity of peptides recognized by T cells (Mwangi et al. 2010). Through normal development, the repertoire of T-cell receptors (TCR) shifts from polyclonal (recognizing many antigens) to oligoclonal (recognizing only a few antigens). This shift is thought to occur through the deletion of T cells that are reactive to food or commensal microbes so as to avoid costly or detrimental immune responses (Probert et al. 2007). However, this process depends on the presence of intestinal microbes, since germ-free rats maintain a polyclonal TCR repertoire, while those inoculated with microbes shift to an oligoclonal repertoire (Helgeland et al. 2004). In mammals, the shift from polyclonal to

oligoclonal could presumably take place during any life stage, as evidenced by the dominance of oral tolerance (uninducibility of the intestinal adaptive immune system by oral antigens) (Friedman 2008). Birds, on the other hand, have a very confined period of developmental oral tolerance, usually only about a week post-hatch (Friedman 2008). Germ-free chickens develop and maintain a polyclonal repertoire early in life (Mwangi et al. 2010). Thus, delayed colonization of microbes may permanently alter the TCR repertoire, causing costly and detrimental immune responses to harmless microbes later on (Probert et al. 2007). Interestingly, the complexity of the microbial community also greatly influences TCR repertoires, suggesting that variation in microbial species colonizing the chicken gut may greatly influence epithelial and systemic immune responses (Mwangi et al. 2010). In mammals, weaning affects the TCR repertoire, presumably through alterations of the microbial community (Probert et al. 2007). Chickens have a constant diet through development, and so it might be informative to investigate TCR patterns in avian species that undergo natural diet shifts during development.

Other components of the gut environment, sometimes considered innate immune defenses, also differ between mammals and birds and can have profound effects on the interactions between commensal microbes and the host. For example, mucins are glycosylated proteins produced by the intestinal tissues that serve as lubricants and protectants of the intestinal epithelium (Johansson et al. 2011). Mucins also provide nutrition and locations for adherence for commensal microbes (Deplancke and Gaskins 2001). These molecules vary in structure between birds and mammals (Verma et al. 1994), resulting in different host–microbe interactions. For instance, chicken mucins, but not human mucins, are able to mitigate the virulent properties of *Campylobacter jejuni*, causing it to assume a commensal role in avian tissues (Byrne et al. 2007). Additionally, it is believed that glycans, oligosaccharides produced by epithelial tissue, regulate microbial communities depending on their diversity and structure (Hooper and Gordon 2001). The presence of certain glycan structures vary between bird species (Ellström et al. 2009), and the avian fucosyltransferase gene important in determining glycan structure has only 50% sequence homology to mammals (Lee et al. 1996). Moreover, avian hosts produce novel defensins, a type of antimicrobial peptide, compared to mammals (Lynn et al. 2004). Allelic variants of avian defensin genes that differ in only several amino acids show functional differences in antimicrobial activities (Hellgren et al. 2010), and so larger differences in sequences between mammals and birds may correspond to functional differences. Together, these differences may regulate gut microbial diversity depending on specificity of their microbial targets and may result in colonization by novel microbes compared to mammals.

Detoxification

Birds consuming plants or invertebrates often ingest secondary metabolites that may act as toxins when absorbed (Karasov and Martinez del Rio 2007). Metabolizing these compounds is energetically expensive, and so it has been proposed that hosts may house detoxifying microbes to save energy (Dearing et al. 2005). Bacteria that degrade saponins have been found in the crop of the hoatzin (Garcia-Amado et al. 2007), and the microbial community of the chicken intestine has been found to metabolize several mycotoxins (Young et al. 2007). However, some microbes also express enzymes that make plant toxins more toxic to the host. For example, many microbes are able to cleave glycosides and glucosinolates, releasing a toxic compound (Hur et al. 2000). These toxic compounds then become more easily absorbed by the host, as shown by an experiment where control chickens absorbed significantly more glucosinolates compared to cecectomized chickens (Slominski et al. 1988). However, there has not been enough research to determine the role of avian commensal microbes in detoxification, or whether microbes play a role in diet diversification over evolutionary time (Dearing et al. 2005).

Additive effects

Microbes in the avian intestinal tract affect nutrition, immunology, and detoxification. However, bacteria seem to have both positive and negative effects in each of these areas, such as liberating nutrients yet decreasing absorption, inducing helpful yet energetically costly immune responses, and either reducing or increasing the toxicity of dietary toxins. Hence, studies with increased host taxonomic diversity must be conducted to elucidate the trade-offs involved in microbial colonization of birds, and specifically which functional roles are most important in terms of individual fitness.

Body temperature

Temperature can influence microbial communities due to differential growth rates and tolerances between microbial species (Mohr and Krawiec 1980). Birds maintain a higher body temperature compared to mammals (Clarke and Rothery 2008). Body temperature also varies between higher groups of birds; ratites show low body temperatures and passerines exhibit some of the highest (Clarke and Rothery 2008). It is likely that the higher body temperature

of birds selects for or inhibits the growth of certain microbial species. This notion is supported by the fact that *Borrelia garinii*, a Lyme's disease-causing agent hosted primarily by birds, is able to grow at higher temperatures compared to mammalian-hosted *Borrelia* species (Hubálek et al. 1998). This is thought to be an adaptation of the microbe to its avian host (Comstedt et al. 2011). However, studies have not yet investigated the role of body temperature in determining gastrointestinal microbial communities.

Variability in intestinal microbes in birds: a potential mechanism for developmental and phenotypic plasticity

Although they are illustrative experimental systems, germ-free animals do not occur in the natural world. Rather, there might be variation in the types and abundances of microbes that colonize individuals of a given host species. In mammals, the gut microbial community is 'inherited' from the mother through contact with fecal and vaginal microbes during the birthing process (Palmer et al. 2007). The importance of this one-time exposure is highlighted by differences in the microbial community structure of conventionally and cesarean-delivered humans from infancy up to at least 7 years of age (Dominguez-Bello et al. 2010; Salminen et al. 2004). Birds however, hatch from eggs, which are presumed to be internally sterile (van der Wielen et al. 2002), and so they may have many different potential sources of microbes. Microbial communities that inhabit eggshells may serve as sources of inoculum and can be modified by parental nesting behavior (Cook et al. 2005; Peralta-Sánchez et al. 2010; Ruiz-De-Castañeda et al. 2011). Additionally, vertical transmission may occur in birds that are fed via regurgitation, in which receiving transferred microbes from their parents is necessary for survival (Godoy-Vitorino et al. 2010; Kyle and Kyle 1993). Juvenile ostriches have been known to engage in consumption of adult feces, which may also aid microbial colonization (Cooper 2004). Yet chickens and turkeys are able to develop normal adult microbiota when hatched completely separately from adults and so they must obtain microorganisms from their surrounding environment (Lu et al. 2003; Scupham 2007). Thus, avian hosts may experience increased variation in the diversity and abundances of microbes that colonize their intestinal tract. This variation may have life-long effects on the phenotype of the host, mediated through altered microbial roles in host physiology discussed above.

With contrasting routes of colonization, the developmental succession of the intestinal microbiota of birds and mammals is also expected to differ. In most mammals and birds, the intestinal microbiota slowly transition to a stable adult-like community. In mammals, large changes in the microbial community structure are observed at points of weaning and transition to solid food (Palmer et al. 2007). However, in developing chickens and turkeys fed a constant diet, large shifts in microbial diversity still occur, presumably due to development of the intestinal environment (Lu et al. 2003; Scupham 2007). Interestingly, the crops of juvenile hoatzins fed by regurgitation have a microbial community with higher diversity compared to chicks and adults (Godoy-Vitorino et al. 2010), suggesting that successional profiles may also differ based on developmental or feeding strategies.

Development of a normal microbial community may be widely influenced by both genetics and environmental variation. For example, in Adelie penguins (*Pygoscelis adelie*), microbial community similarity is negatively correlated with both host genetic distance and geographic distance (Banks et al. 2009). Furthermore, a cross-foster experiment between great tits (*Parus major*) and blue tits (*P. caeruleus*) revealed that the environmental factors associated with a shared nest were more important than host species in determining microbial community structure (Lucas and Heeb 2005). However, at some point avian host genetic differences can have larger effects on the microbiota. For example, nestlings of a brood parasite, great spotted cuckoos (*Clamator glandarius*), and their host, magpies (*Pica pica*), sharing the same nest and parents have drastically different microbial communities (Ruiz-Rodríguez et al. 2009a). In mammals, host phylogeny seems to determine the intestinal microbial community more than environmental factors, as shown by similar microbial communities within mammalian host species housed in different zoos or even different continents (Ley et al. 2008a).

Environmental variation in the intestinal microbial community may have long-term effects on the developing avian and mammalian hosts. For example, artificial environmental variation in mammalian microbes was conducted by colonizing germ-free rabbits with mouse cecal microbes. These mouse-colonized rabbits have decreased body mass, lower digestibility, and are more susceptible to disease compared to those colonized with rabbit cecal microbes (Boot et al. 1985). However, in mammals, the strong vertical transmission of microbes reduces natural variation in community composition. Thus, in birds, natural selection may act on the parental behaviors which inoculate nestlings with optimal intestinal microbes (Soler et al. 2010). Abundances of certain intestinal microbes in nestlings have been correlated with several host phenotypic conditions such as wing asymmetry (Mills et al. 1999), nestling size (Moreno et al. 2003), body condition, and immune responses (Ruiz-Rodríguez et al. 2009b),

suggesting a significant role of specific intestinal microbes in avian development. In mammals, exposure to microbes early on can have developmentally plastic (i.e., irreversible) effects on phenotypic parameters such as immune and stress responses (Boissé et al. 2004; Shanks et al. 2000). These parameters have also been shown to be developmentally plastic in birds (Love and Williams 2008; Pitala et al. 2007), but the role of microbes in their development remains unclear.

Additionally, birds may experience variation in intestinal microbes as adults. Adult passerine birds show differences in their microbial communities due to geographic location, diet, and season (Klomp et al. 2008; Maul et al. 2005). These differences may simply be due to shifts in relative abundances of microbial species, and not necessarily inoculation of new microbes. However, as the cloaca serves as both an excretory, as well as copulatory organ, microbes can be transmitted between mates during sexual contact (White et al. 2010). Inoculation of new microbial species may cause host phenotypic variation through altered microbial roles in nutrition or immunity, and thus females may select for males with certain microbial assemblages (White et al. 2010). Though, in order for sexually transmitted microbes to colonize the adult avian intestinal tract, an immune response must be avoided. Developing oral tolerance is the most common way to avoid an immune response to new microbes, but this period, measured in chickens, is only a week in duration (Friedman 2008). Introduction of new microbes and their avoidance of an immune response in passerine birds clearly demands future study.

Future directions

16S rDNA sequence inventories

Many studies on the intestinal microbial communities of avian hosts use methods that underrepresent diversity. The majority of studies presented in this review used culture-based or molecular fingerprint techniques to correlate microbial diversity with various phenotypic traits. However, both of these techniques are known to underestimate microbial abundance and diversity. Culture-based experiments usually focus on microbial taxa of interest and cannot detect the 99% of microbes estimated to be unculturable. Molecular fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) and automated ribosomal intergenic spacer analysis (ARISA) make use of differences in sequence integrity or length between microbial taxa to create molecular banding patterns, which can be used to characterize a community. However, similarities in sequence integrity or length between members of disparate microbial taxa often result in large underestimates of diversity (Fisher and Triplett 1999; Smalia et al. 2007).

In order to more finely detect differences in microbial communities, investigators should rely on molecular cloning and sequencing of the 16S rDNA gene. The 16S rDNA sequence has a slow rate of change, is rarely transferred between microbial species, and is of sufficient size for bioinformatic and phylogenetic analysis, making sequence analysis a robust method for the characterization of microbial diversity (Head et al. 1998). To date, 16S rDNA inventories have only been conducted on 10 avian species (Table 1). There have been many microbial inventories of mammalian intestinal microbes, including one study alone that inventoried 60 mammalian species (Ley et al. 2008a). Additionally, advances in pyrosequencing now allow for large-scale inventories at relatively low costs (Dowd et al. 2008), but with increased error rates and shorter sequence lengths. Due to the potential novelty of avian microbes, researchers should also continue to create full-length, robust sequence libraries via Sanger sequencing.

Meta-"omics"

Due to the wide variation in genome content between even closely related strains of microbes (Nelson et al. 2010), it is difficult to assign putative functions to microbial populations based on 16S sequence inventories alone. To better deduce microbial functions, researchers now use metagenomic sequencing. This technique employs high-throughput, non-specific microbial DNA sequencing to inventory the many genes present in an environmental sample, and allows researchers to compare microbial functional diversity rather than solely taxonomic diversity. Recovered gene sequences can be assigned to functional categories (carbohydrate metabolism, membrane transport, xenobiotic metabolism, etc.), and the representation of different functional categories can be directly compared between samples. To date, metagenomic sequencing has only been conducted on the domesticated chicken (Qu et al. 2008).

Metagenomic sequences represent only the 'potential' of a microbial community and not necessarily the actual function. To monitor what genes are expressed, researchers now utilize metatranscriptomics and metaproteomics. Metatranscriptomics utilizes similar technology as metagenomics, but instead sequences microbial mRNA transcripts (via cDNA). However, differences in transcript abundances could be due to changes in either gene expression or different levels of microbial representation. To circumvent this problem, researchers must conduct a parallel metagenome to normalize abundances with transcript:gene ratios. Metaproteomics compares protein

abundances between environmental samples by separating proteins and sequencing those that have differential representation. However, due to limitations in technology, metaproteomics often focuses only on these differentially represented proteins (Wilmes and Bond 2006), making it useful for comparative studies, but not for gaining insight into the function of a whole microbial community.

There has also been interest in developing meta-metabolomics, the inventory of small molecule networks existing in an environmental sample (Turnbaugh and Gordon 2008). This technique involves identification of metabolites using nuclear magnetic resonance (NMR) or mass spectrometry (MS). In terms of microbe–host interactions, it is perhaps most relevant to investigate the host metabolome, because these profiles affect host physiology. For example, it has been found that colonization by microbes strongly influences the metabolomic profile of mammalian blood (Wikoff et al. 2009), but this has not yet been studied in avian species.

Physiological performance

Meta-"omics" approaches are often conducted to understand the staggering complexity of intestinal microbial communities. However, these metrics alone only allow insight into the potential metabolic capabilities of the microbiota. Therefore, investigators should continue to pursue other experimental methods to determine the functions of avian microbial communities and their roles in physiological performance.

Removal of microbial communities by antibiotic treatment, followed by nutrient supplementation has been used extensively to understand the function of microbial symbionts in insects. For example, microbe-free aphids show reduced growth and survival on diets lacking certain essential amino acids, while aphids with symbionts are less affected (Dadd and Krieger 1968; Mittler 1971). Likewise, bedbugs treated with antibiotics show decreased growth and survival, but these effects are reduced when diets are supplemented with B vitamins (Hosokawa et al. 2010). Microbial colonization is thought to be necessary for the growth and survival of passerine birds (Kyle and Kyle 1993), but nutrient supplementation of young birds hatched in sterile environments might reveal specific nutritional roles of the avian microbiota. In addition, there are bird species (e.g., hummingbirds, sunbirds) that feed on nutritionally incomplete diets. Insects feeding on such diets possess vertically transmitted microbes that are critical to their survival (Mittler 1971). It is possible that birds too have such obligate symbionts.

The use of germ-free organisms has also furthered the understanding of how microbial communities influence host physiology. Comparisons of germ-free and conventionally raised birds have been used to investigate numerous physiological processes (discussed above), but not all potential processes have been studied (stress response, behavior, etc.). Additionally, gene expression patterns in the intestines (Hooper et al. 2001) and livers (Claus et al. 2011) vary between conventionally raised and germ-free mice. However, similar gene expression studies using germ-free and conventional birds have not been conducted.

Stable and radioactive isotopes are additional tools for tracking compounds or nutrients of interest in microbial communities. One method for this technique is to expose complex microbial communities to labeled substrates. Microbes that are able to utilize the substrates of interest incorporate the labeled atoms into their DNA, and this 'heavy', labeled microbial DNA can easily be separated from other DNA by density gradient centrifugation. Isolated DNA can then be functionally or taxonomically characterized by sequence analysis (Radajewski et al. 2000). Labeled compounds have also been used to investigate the rates of oxidation of various nutritional compounds in birds (McCue et al. 2010). Comparing the fates and oxidation rates of labeled nutrients, toxins, and other compounds between conventional and germ-free birds would help to elucidate what role microbes play in the routing of nutrients and toxins.

Integrative approaches and utilizing host taxonomic diversity

Simultaneously conducting 16S inventories, meta-"omics" and physiological performance assays synergistically advances the knowledge gained in a single experiment. For example, correlating the abundance of a microbial species (based on 16S inventories) with host gene expressions or physiological assays can lead to hypotheses of how abundances of certain microbes influence host physiology (Claus et al. 2011).

Additionally, future research should embrace the diverse physiological strategies of avian hosts. Much of what we know about interactions between microbes and avian hosts are derived from studies on domesticated chickens. However, dietary strategies and gut anatomies vary widely between avian taxa. Pigeons (Columbiformes), parrots (Psittaciformes) and many fowl (Galliformes) are all granivorous, yet only Galliformes maintain cecal chambers that house microbes (DeGolier et al. 1999). Similarly, eagles, hawks, and falcons (Falconiformes) and owls (Strigiformes) share similar carnivorous diets, yet only owls maintain ceca (DeGolier et al. 1999). Comparative approaches between taxa will illustrate how dietary strategy, gut anatomy, as well as how variation in microbial communities might influence host–microbe relationships in avian taxa.

Summary

Our knowledge of the role of intestinal microbes in avian hosts lags far behind our understanding of mammalian systems. Studies that have been done, mostly in chickens, show that intestinal microbes play large roles in terms of host nutrition, immunity, and development. Researchers should now embrace host phylogenetic diversity, as well as quickly advancing methods to study avian intestinal microbial ecology. With a broader knowledge of avian hosts, we may be able to compare and contrast solutions of terrestrial vertebrate hosts to various physiological challenges, perhaps gaining insight into the evolution of the intestinal microbiota in a broader sense.

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APPENDIX B

RESTRUCTURING OF THE AMPHIBIAN GUT MICROBIOTA THROUGH METAMORPHOSIS

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Restructuring of the amphibian gut microbiota through metamorphosis

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Summary

Vertebrates maintain complex symbioses with a diverse community of microbes residing within their guts. The microbial players in these symbioses differ between major taxa of vertebrates, such that fish and amniotes maintain notably different communities. To date, there has not been a culture-independent inventory of an amphibian gut microbial community. Here, we compared gut microbial communities of tadpoles and frogs of the Northern leopard frog (*Lithobates pipiens*). We utilized Illumina sequencing, which allowed us to inventory more than 450 000 microbial sequences. We found that tadpoles and frogs differ markedly in the composition of their gut microbial communities, with tadpoles maintaining a community more similar to fish, whereas the frog community resembles that of amniotes. Additionally, frogs maintain a community with lower phylogenetic diversity compared with tadpoles. The significant restructuring of the microbiota is likely due to changes in diet as well as the large reorganization of the intestinal organ during metamorphosis. Overall, we propose that amphibians represent an important system in which to study regulation and selection of gut microbial communities.

Introduction

Symbioses between animals and microbes have markedly influenced the ecology and evolution of both players (McFall-Ngai *et al.*, 2013) by modulating energy balance (Semova *et al.*, 2012), immune function (Round and Mazmanian, 2009) and even behaviour (Heijtz *et al.*, 2011) of the host. Though these symbioses are ubiquitous, the microbial communities residing within the vertebrate gut differ largely among host phylogenetic classes. Teleost fish host communities are rich in *Proteobacteria* (Rawls *et al.*, 2006; Sullam *et al.*, 2012), while previously studied amniotes (mammals, birds and diapsid reptiles) maintain communities dominated by *Firmicutes* and *Bacteroidetes* (Ley *et al.*, 2008; Scupham *et al.*, 2008; Costello *et al.*, 2010). These differential communities seem to be selected by the host, though the mechanisms are still unclear (Rawls *et al.*, 2006). Amphibians represent an important, intermediate clade between these groups (Kardong, 1995); yet to date, there has not been a culture-independent inventory of an amphibian gut microbiota.

Amphibians undergo many physiological and morphological changes through development. Anuran tadpoles lack fully developed external appendages, breathe with gills and are fully aquatic. Through metamorphosis, frogs complete development of limbs, gain the ability to breathe air and may adopt terrestrial lifestyles. The diets of amphibians also largely change over metamorphosis. As tadpoles, many species consume diets comprised almost entirely of plant material, whereas frogs are primarily insectivorous (Jenssen, 1967; Linzey, 1967; Hendricks, 1973). The digestive tract also undergoes rapid and radical changes between these life stages, from non-acidic stomachs and reduced hindguts in tadpoles, to acidic stomachs, typically shorter small intestines and enlarged hindguts in adults (Stevens and Hume, 1995; Hourdry *et al.*, 1996). Likewise, the immune system of the gut is underdeveloped in tadpoles compared with metamorphosed frogs (Du Pasquier *et al.*, 2000). These developmental changes may have large implications for determining the microbial community that resides within the guts of tadpoles compared with frogs.

Here, we compared gut microbial inventories of tadpoles and frogs of the northern leopard frog (*Lithobates pipiens*). This study represents the first culture-independent investigation of the gut microbial community of an amphibian. Tadpoles were fed a diet of ground alfalfa (88% of dry mass) suspended in a matrix of agar and gelatin (12%) *ad libitum*, while those allowed to develop through metamorphosis were fed a diet of crickets and mealworms for 16 weeks as frogs. We collected total digesta from the whole intestine (small and large) of tadpoles and frogs and conducted microbial inventories by sequencing the 16S rRNA gene on an Illumina MiSeq platform (Illumina Inc., San

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Table 1. Relative abundances (mean ± SEM) of major bacterial phyla residing in the guts of tadpoles ($n = 7$) and frogs ($n = 8$). *P*-values were calculated with a Student's *t*-test. Significant differences are in bold.

	Tadpoles	Frogs	*P-value*
Firmicutes	36.61 ± 8.08	66.05 ± 8.90	**0.029**
Proteobacteria	54.86 ± 7.55	10.43 ± 3.39	**0.0006**
Bacteroidetes	2.43 ± 1.28	22.82 ± 8.96	0.057
Verrucomicrobia	2.66 ± 0.80	0.03 ± 0.01	**0.016**
Actinobacteria	1.13 ± 0.51	0.08 ± 0.06	0.084
Tenericutes	0.95 ± 0.25	0.03 ± 0.02	**0.011**
Planctomycetes	0.47 ± 0.34	< 0.01	0.21
Fusobacteria	< 0.01	0.32 ± 0.11	**0.028**
Acidobacteria	0.09 ± 0.04	0	0.051

Diego, CA, USA) (Caporaso *et al.*, 2012). We predicted that the microbial community structure would vary across developmental stages because of the considerable changes in diet and intestinal morphology/physiology between these groups.

Results and discussion

A total of 462 947 high-quality microbial 16S rRNA sequences were produced through Illimuna sequencing of the gut contents of tadpoles and frogs (13 930 ± 403 sequences per sample). These sequences were classified into 7908 operational taxonomic units based on 97% sequence identity using QIIME (Caporaso *et al.*, 2010). Sequences were deposited in GenBank under accession SRP019766. Details regarding animal collection, sequencing and data analysis can be found in Supplementary Methods (Fig. S1).

The anuran gut microbial community exhibited marked differences between tadpole and frog life stages. Relative abundances of five of the nine most dominant phyla in the anuran gut differed significantly with life stage (Table 1). Tadpoles harboured a community dominated by the phyla *Proteobacteria* and *Firmicutes*, while frogs maintained a community rich in *Firmicutes* and *Bacteroidetes*. Through development from tadpoles to frogs, anurans exhibited significant reductions in the relative abundances of *Proteobacteria*, *Verrucomicrobia*, *Tenericutes*, and showed trends for reduction in the abundance of *Actinobacteria* and *Acidobacteria* (Table 1). The phylum *Acidobacteria* was present only in tadpoles, and absent from all frogs. Through metamorphosis, there was also a significant increase in the relative abundance of *Firmicutes* and *Fusobacteria*, as well as a trend for *Bacteroidetes* to increase in abundance (Table 1).

Interestingly, these life stages were more similar to communities observed in disparate host taxa rather than to one another. Tadpoles maintained a community dominated by *Proteobacteria*, which is similar to gut communities harboured by teleost fish (Sullam *et al.*, 2012). Conversely, frogs housed a community rich in *Firmicutes* and *Bacteroidetes*, which is more similar to communities in amniotes (Ley *et al.*, 2008; Scupham *et al.*, 2008; Costello *et al.*, 2010). The phylum *Acidobacteria* was detected in roughly half of the tadpole digesta samples, but was undetectable in all frog samples. This transition mirrors differences between fish, which usually harbour *Acidobacteria* (Sullam *et al.*, 2012), and amniotes, where *Acidobacteria* are generally undetectable (Ley *et al.*, 2008; Costello *et al.*, 2010). Recently, Costello *et al.* (2010) conducted the first large-scale inventory of a reptile gastrointestinal microbial community and showed that a *Firmicutes*- and *Bacteroidetes*-rich gut community in adult individuals is a trait of amniotes. Our results represent the first inventory of an amphibian, and suggest that tetrapods in general share this trait.

These trends are further supported when comparing microbial genera specific to certain developmental stages of the northern leopard frog. Tadpole- and frog-specific genera were defined as those that were detected in more than half of the individuals of one group, and completely absent from all samples of the other group (Table 2). Several tadpole-specific genera (e.g. *Shewanella, Hydrogenophaga, Devosia*) are predominant members of invertebrate or fish microbial communities (Grossart *et al.*, 2009; Li *et al.*, 2009; Navarrete *et al.*, 2009), while frog-specific genera (e.g. *Odoribacter, Butyricimonas, Akkermansia*) are largely found in the guts of amniotes (Derrien *et al.*, 2008; Sakamoto *et al.*, 2009; Nagai *et al.*, 2010). It is worth noting that although tadpoles had a higher proportion of microbes belonging to the phylum *Verrucomicrobia*, they lacked the frog-specific genus *Akkermansia* (a member of *Verrucomicrobia*). Rather, this phylum-level difference was due to tadpoles harbouring a higher proportion of unidentified microbes belonging to the family *Verrucomicrobiaceae*. A larger survey of amphibian species, especially across various orders, is warranted to investigate the generality of these differences between developmental stages.

Table 2. Tadpole- and frog-specific genera, defined as those that were detected in more than half of the individuals of one group, and completely absent from all samples of the other group.

Genera found only in tadpoles	Genera found only in frogs
Paenibacillus	*Odoribacter*
Novosphingobium	*Butyricimonas*
Hydrogenophaga	*Dysgonomonas*
Shewanella	*Akkermansia*
Devosia	
Rheinheimera	
Emticicia	
Flectobacillus	
Gemmata	
Aminobacter	

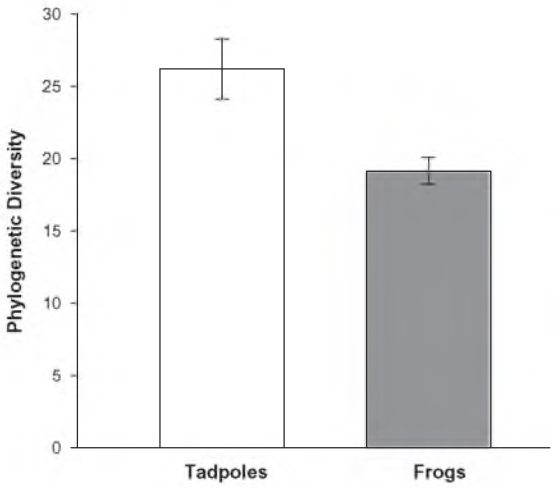

Fig. 1. Faith's phylogenetic diversity index of tadpole and frog gut microbial communities.

Indices of microbial diversity in the gut also differed between tadpoles and frogs. Frogs harboured a community with significantly lower Faith's phylogenetic diversity index than tadpoles ($P = 0.014$, Fig. 1). However, there was no difference in estimated species richness, evenness or the Shannon Index between tadpoles and frogs ($P > 0.25$ for all). Tadpoles and frogs maintained microbial communities with different composition as indicated by Principal Coordinates Analysis of unweighted UniFrac data (Fig. 2). These differences are unlikely to be solely driven by diet, as food sources and water contained very different microbial communities than the anuran gut (Fig. S1).

The change in diet between tadpoles and frogs may drive the observed changes in communities between these two developmental stages. Tadpoles are primarily herbivorous, while frogs are typically insectivorous. Thus, frogs consume a diet higher in protein and chitin, and lower in cellulose compared with tadpoles. Dietary strategy determines microbial community structure in mammals such that herbivores and carnivores have unique communities (Ley *et al.*, 2008). Additionally, changes in content of plant polysaccharides can influence microbial community composition (Turnbaugh *et al.*, 2009). In this baseline study, we aimed to maintain tadpoles and frogs on their typical diets. Future studies may wish to use similar, artificial diets through development to parse out the role of diet on community structure of microbes.

In addition to diet, host factors may select which microbial members flourish within the gut, though the mechanisms are still unknown. When germ-free zebrafishes are inoculated with a *Firmicutes*-rich, mammalian microbial community, the introduced microbial community is resculpted to one rich in *Proteobacteria* (Rawls *et al.*, 2006). Simple physicochemical differences between tadpoles and frogs may explain this difference in community structure. *Proteobacteria* exhibit an increased tolerance to oxygen compared with *Firmicutes* and *Bacteroidetes* (Rawls *et al.*, 2006). Additionally, development of a gastric stomach (absent in tadpoles and present in frogs) and changing gut pH through metamorphosis (Stevens and Hume, 1995; Hourdry *et al.*, 1996) likely alter the microbial community (Duncan *et al.*, 2009). Through metamorphosis, amphibians also undergo rapid degeneration of the flat, primary intestinal epithelium and proliferation of a secondary intestinal epithelium (Hourdry *et al.*, 1996). This secondary epithelia exhibits folded villi, and higher expression of many digestive and innate immunity genes (Hourdry *et al.*, 1996). Epithelial immune function also changes through amphibian metamorphosis, such that the larval gut lacks B cells producing IgM or IgX (Mussmann *et al.*, 1996; Du Pasquier *et al.*, 2000). The types of glycoconjugates produced by the small intestine changes through metamorphosis (Kaptan *et al.*, 2013), which may facilitate colonization by certain microbe species by providing energy sources or binding areas (Hooper and Gordon, 2001). This is further supported by the fact that *Akkermansia*, a genus that specializes on intestinal mucins (Derrien *et al.*, 2008), is found only in frogs and absent from tadpoles. It is likely that these intricate changes in gene expression between tadpoles and frogs result in shifts in microbial diversity. Further studies are necessary to investigate these hypotheses.

Significant restructuring of the gut microbiota has been observed in other systems. The gut microbiota is repeatedly remodelled in pythons during fasting (Costello *et al.*, 2010) and in 13-lined ground squirrels during hibernation (Carey *et al.*, 2013). The guts of several species of insect undergo sterilization and recolonization through metamor-

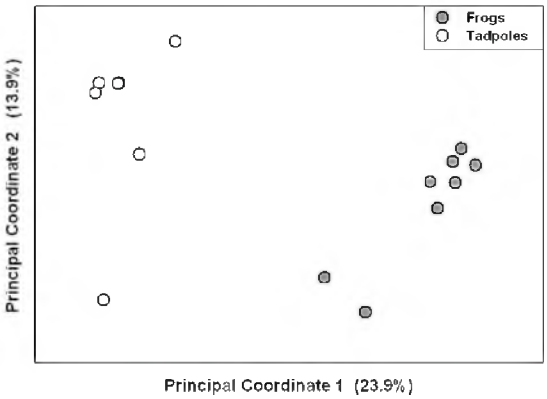

Fig. 2. Principal Coordinate Analysis using unweighted UniFrac scores of the microbial communities from tadpoles and frogs.

phosis because of production of a cocktail of antimicrobial compounds (Russell and Dunn, 1996; Moll *et al.*, 2001; Koch and Schmid-Hempel, 2011). Similarly, amphibians produce high levels of lysozymes with antimicrobial activity during the climax of metamorphosis (Hourdry *et al.*, 1996). Future studies could conduct microbial diversity and density measurements at various time points throughout metamorphosis to gain better insight in to this process.

Overall, we documented large changes in microbial diversity between tadpoles and frogs. We found that tadpoles and frogs differ significantly in the composition of their gut microbial community, with tadpoles maintaining a community more similar to fish, and frogs resembling amniotes. The changes in diet and gastrointestinal physiology between tadpoles and frogs make amphibians an ideal study system in which to study the host regulators of microbial diversity at the phylum level. Although this study only monitored changes in community structure, the results raise a number of questions and hypotheses that should be addressed to advance our understanding of the mutualisms between vertebrate hosts and gut microbes. Additionally, the physiological, ecological and evolutionary roles of these disparate communities remain to be investigated.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Principal Coordinate Analysis using unweighted UniFrac scores of the microbial communities from tadpoles and frogs, as well as food and water sources.
Appendix S1. Supplementary methods.